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13. ABSTRACT (Maximum 200 Words) The purpose of this proposal is to develop a "next generation" representational difference analysis that uses transcribing nuclear DNA. Organomercury and Sulfolink Coupling gel chromatography could isolate soluble transcribed chromatin from breast cancer cells. However, most transcribed chromatin is insoluble. Thus, we used ChIPs (chromatin immunoprecipitation) technique to isolate active chromatin that is bound to highly acetylated histones. Before applying ChIPs, we studied the dynamics of histone acetylation in breast cancer cells, and the effect of estradiol on these processes. The kinetics of histone acetylation in hormone dependent (T47D5) and hormone independent (MDA MB 231) breast cancer cells were determined. Estradiol increased histone acetylation by decreasing the rate of histone deacetylation in T47D5 cells. Estradiol had no effect on histone acetylation in hormone independent cells. The mechanism by which estradiol decreased the rate of histone deacetylation was explored. Estradiol affected the subnuclear trafficking of the estrogen receptor (ER) and ER associated coactivators (SRC-1 and SRC-3, which have histone acetyltransferase activity) which become tightly bound to the nuclear matrix. However, estradiol did not affect the activity or subnuclear distribution of histone deacetylases. Finally, the ChIPs protocol was successfully used to isolate transcriptionally active DNA from T47D5 and MDA MB 231 cells.				
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4. INTRODUCTION

The goal of this research is to establish a protocol to isolate transcriptionally active chromatin from human breast cancer cells. The strategy of this protocol exploits features of transcriptionally active chromatin. As transcribed chromatin is associated with highly acetylated histones [1-5], we exploited this feature of active chromatin using a chromatin immunoprecipitation (ChIPs) technique [4,6-8]. Before embarking with the ChIPs procedure, it was important to understand the dynamics of histone acetylation in breast cancer cells, and the effect of estradiol on this process. At the time that we initiated these studies, there was one report that showed that estradiol administration to hormone dependent breast cancer cells significantly reduced the level of acetylated histones [9]. As rates of histone acetylation and deacetylation in human breast cancer cells have not yet been reported, we did an in depth analyses of the dynamics of histone acetylation in hormone dependent (T47D5) and hormone independent (MDA MB 231) breast cancer cell lines and the effect that estradiol had on these processes. We found that estradiol increased the steady state level of histone acetylation in estrogen receptor positive but not estrogen receptor negative breast cancer cells. Further analyses revealed that estradiol affected the rate of histone deacetylation but not the rate of acetylation. The decreased rate of deacetylation twenty minutes following the addition of estradiol explained the increase in steady state levels of histone acetylation.

In this report we explored the mechanism by which estradiol decreased the rate of histone acetylation. We found that estradiol does not have an affect on histone deacetylase activity, histone deacetylase subnuclear trafficking or turnover. However, estradiol did affect the subnuclear trafficking of the estrogen receptor and estrogen receptor associated transcriptional coactivators, which have histone acetyltransferase activity. Based on these results, we have proposed a model that explains the observed decreased rate of histone deacetylation in estradiol treated estrogen receptor positive breast cancer cells. We also demonstrate that the ChIPs protocol with antibodies against acetylated H3 or H4 histones is an effective method to isolate transcriptionally active DNA from human breast cancer cells.

5. BODY OF REPORT

A. EXPERIMENTAL METHODS

i. Cell Lines and Culture Conditions

Human breast cancer cell line T47D5 (ER positive and hormone dependent) and MDA MB 231 (ER negative and hormone independent) were grown in DMEM (GIBCO) medium supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and 5% glucose. For the ligand treatment experiments, cells were grown in phenol red free DMEM, containing 7% charcoal stripped FBS for 3 days. All cells were grown in a 37°C humidified incubator with 5% CO₂. Different batches of T47D5 and MDA MB 231 cells were continually grown and used in the experiments.

ii. Preparation of Cell Lysate, Cytosol, Nuclei and Triton X-100 Fractions

T47D5 cells were resuspended in TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1% thioglycol) containing 1 mM PMSF and protease inhibitor cocktail (Roche). The cells were lysed by passing through a #22 gauge needle. The cytosol and nuclei were isolated from lysed cells after centrifugation at 4500 x g. Preparations of nuclei were inspected under the microscope. The nuclei were resuspended in TNM buffer. Half of the nuclei preparation was extracted by adding Triton X-100 to a final concentration of 0.5%, and incubated on ice for 5 minutes. After centrifugation at 4500 x g for 10 minutes, the supernatant, named the Triton X-100 supernatant fraction or Triton-S, was saved. The nuclei pellet was resuspended in an equal volume of TNM buffer with 0.5% Triton X-100; this fraction was named the Triton X-100 pellet fraction or Triton-P.

For Western blot analysis, equal volumes of cell lysate, cytosol, nuclei, Triton-S and Triton-P were diluted with the same volume of SDS sample buffer (125 mM Tris-HCl, pH 6.5, 3% SDS, 5% β-mercaptoethanol and 10% glycerol) then an equal volume of 2x SDS loading buffer was added. After boiling for 5 minutes, the samples were loaded onto a SDS 8% or 10% polyacrylamide gel, and transferred onto nitrocellulose membranes.

For HAT and HDAC activity assay, 20 µl of the cell fraction was used to perform enzyme activity assay. To determine background radioactivity, the assay was done with

an equal volume of the fraction that was boiled for 5 minutes to destroy the enzyme activity.

iii. Pulse-Chase Labelling Cells and Ligand Treatment of Cell

MDA MB 231 cells were pulse-chase labelled with [^3H]-acetate and incubated with estradiol as described previously (1999 annual report, Methods).

iv. Western Blot Analysis

Western blot analysis was carried out as described previously (1999 annual report, Methods). Polyclonal antibodies against human HDAC1 (Affinity Bioreagents Inc., ABR), human HDAC2 (ABR), human HDAC3 (ABR), human HDAC4 (Santa Cruz), human SRC-1 (ABR), mammalian Sp1 (Santa Cruz), mammalian Sp3 (Santa Cruz), and mouse monoclonal antibodies against human ER α (Novocastra Laboratories Ltd), SRC-3 (AIB1/RAC3/ACTR) (Upstate) were used.

v. Immunoprecipitation

T47D5 cells were lysed in Low Stringency IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM EDTA and protease inhibitor cocktail) and sonicated twice for 15 seconds at 30% output setting on the sonicator (Sonifier Cell Disrupter). After centrifugation at 12000 x g for 10 minutes, the supernatant, named the cell lysate, was kept. The cell lysate was diluted with IP buffer to 2 A₂₆₀/ml. The antibodies were added to the cell lysate, and incubated at 4°C for 16 hours. After adding protein A-Sepharose, the cell lysate was incubated for 2 hours at 4°C. Protein A-Sepharose was collected by centrifugation and washed four times with IP buffer. Some immunoprecipitates were used for Western blotting analysis. SDS loading buffer was added to the protein A-Sepharose, and boiled for 5 minutes. The sample was loaded onto SDS 10% polyacrylamide gels, and transferred onto nitrocellulose membranes and subsequently immunochemically stained. Other immunoprecipitates were used for HDAC activity assays where the protein A-Sepharose was resuspended in a suitable buffer.

vi. HDAC and HAT Activity Assay

HAT activity assay was done as described previously [10]. In brief, 20 μ l of sample was added to a microcentrifuge tube containing 0.5 μ Ci of [3 H]-acetyl-coenzyme A (NCI), 100 μ g of total chicken core histones and 1x HAT buffer (50 mM Tris-HCl, pH 7.5, 50 mM sodium butyrate, 15 mM β -mercaptoethanol) to a final reaction volume of 150 μ l. The reaction was incubated at 37°C for one hour. The reaction was spotted onto p81 paper (Whatman), and washed with 50 mM sodium carbonate (pH 9.1). The paper was counted in a liquid scintillation counter (Beckman). As controls, an equal volume of the samples, which were boiled for 5 minutes to destroy enzyme activity, was assayed. Radioactive counts from the control assay were subtracted from those of the test sample to calculate the net counts of the sample. HDAC activity assays were performed as described previously (1999 annual report, Method).

vii. Chromatin Immunoprecipitation (ChIPs) and PCR Analysis

The ChIPs protocol was described previously (1999 annual report, Methods). The flow chart of ChIPs technique is shown in Fig. 14. DNA fragments isolated by ChIPs or from the input were used as a template in PCR reactions. Primers were human ER α -exon I (upstream 5'-TTCGTCCTGGGAGCTGCACTT-3' and downstream 5'-GCAGAAGGCTCAGAAACCGGC-3'), human *c-myc* exon I (upstream 5'-GAGCTGTGCTGCTCGCGGCCGCA-3' and downstream 5'-CCCTATTCGCTCCGGATCTCCCTT-3') and human GAPDH exon VII (upstream 5'-AAGGTCATCCCTGAGCTGAAC-3' and downstream 5'-CCAGGAAATGAGCTTGACAAA-3'). PCR was carried out as previously described [11].

B. ASSUMPTIONS

The goal of this project is to isolate transcriptionally active chromatin from T47D5 cells (ER positive and hormone dependent) and MDA MB 231 cells (ER negative and hormone independent). A "next generation" representational difference analysis (RDA) was designed to detect "all" transcribed genes including those that code for strictly nuclear RNA. To accomplish this task, we used the ChIPs protocol to isolate

transcriptionally active chromatin. Our strategy was to isolate transcribed DNA sequences, which were associated with acetylated histones. Before embarking on this process, we did an in-depth study on the effect of estradiol on the dynamics of histone acetylation in human breast cancer cells. In the past year, we investigated the mechanism by which estradiol alters the histone acetylation status of breast cancer cell chromatin.

We hypothesize that estradiol affects the sub-cellular distribution of HATs and HDACs. Dependent on ligand, ER is associated with coactivators with HAT activity or corepressors with HDAC activity. When estradiol is bound to ER, coactivators with HAT activity are recruited to estrogen responsive promoters, resulting in acetylation of histones at that site [12,13]. Thus, estradiol-ER alters the histone acetylation status of breast cancer cell chromatin.

Since highly acetylated histones are associated with the transcriptionally active DNA, anti-acetylated H4 and anti-acetylated H3 antibodies could be applied to immunoprecipitate transcriptionally active chromatin. It has been reported that acetylation of H3 and H4 at specific chromatin sites may not be equivalent, that is, acetylation of H3 may be more extensive than that of H4 [14]. Thus, we tested each antibody for its capability to immunoprecipitate transcribed DNA.

C. RESULTS AND DISCUSSION

The goal of this project is to identify genes that are differentially transcribed in human breast cancer cells and tumors. A protocol was designed to isolate transcribed chromatin using either an affinity chromatography or a ChIPs technique. The transcribed chromatin from human breast cancer ER positive and ER negative cells would be analyzed by representational difference analysis. We expected that this protocol would detect all transcribed genes aberrantly transcribed during the progression of breast cancer. In the first-year of study, organomercury chromatography and Sulfolink Coupling gel chromatography were performed to isolate transcribing chromatin from human breast cancer cells. Since the insoluble chromatin fraction contained most of the transcribed DNA and the organomercury column was discontinued commercially, an alternate protocol, chromatin immunoprecipitation, was

designed to isolate the transcribed chromatin, which was associated with highly acetylated histones. Before embarking with the ChIPs procedure, we investigated the dynamics of histone acetylation in breast cancer cells in the second year of study. The experiments showed that estradiol increased the steady state level of histone acetylation by reducing the rate of histone deacetylation. In the third-year study, the mechanism by which estradiol altered the steady state level of histone acetylation was investigated. Immunoprecipitation and Western blot analyses showed that estradiol did not affect the level, subnuclear distribution or activity of HDAC. However, estradiol did play a role in the sub-cellular distribution of ER and ER-associated coactivators, which have HAT activity. The experiments showed that ER-HDAC1, Sp1-HDAC1 and Sp3-HDAC1 complexes occur in T47D5 cells. After 20 minutes treatment, estradiol reduced HDAC activities in ER-HDAC complexes; however, estradiol did not affect HDAC activity in the Sp1-HDAC complex. Based upon the knowledge of histone acetylation of human breast cancer cells, the ChIPs protocol was used to isolate the transcribed chromatin from human breast cancer ER positive and ER negative cells. The RDA procedure has not yet been done. However, using funds from a Canadian Institute of Health Research operating grant, we will continue this project.

i. Isolation of Transcriptionally Active Chromatin from Human Breast Cancer Cells Using Organomercury Column and Sulfolink Coupling Gel Chromatography

In the first-year study, organomercury column and Sulfolink Coupling gel column chromatography were used to isolate transcribed chromatin. In mammalian histone H3, cysteine is found at position 110. In most nucleosomes, this cysteine residue is buried and not available to react with thiol reactive reagents. In the transcribing nucleosome, this cysteine is exposed and is available to react with thiol reactive reagents such as mercury and iodoacetyl groups.

Chromatin fractions were prepared from human breast cancer cells (1998 annual report, Result ii). Electrophoretic analyses of histones and Southern blot analyses of chromatin fractions showed that most transcriptionally active chromatin was associated with the nuclear matrix PE fraction (1998 report, Fig. 3, 4). Organomercury column chromatography was performed to isolate the transcribed chromatin from soluble

chromatin fractions (S0 and SE). Western blot and Southern blot analyses showed that the mercury column can enrich for transcribed chromatin. However, the manufacturer of this column (BioRad) has discontinued this product. We developed a novel procedure using Sulfolink Coupling gel (Pierce) chromatography to continue this study.

The Sulfolink Coupling gel contains immobilized iodoacetyl group that can react with sulfhydryl groups. The different chromatin fractions (soluble S0 and SE) of breast cancer cells were applied to Sulfolink Coupling gel column. Different wash conditions were tested (1998 report, Fig.6). Southern blot analyses of DNA fragments from the bound fraction showed that transcribed chromatin was associated with the Sulfolink Coupling gel (1998 report, Fig. 7).

The organomercury column and Sulfolink Coupling gel column chromatography procedure worked for isolation of transcribed chromatin from soluble chromatin fractions (S0 and SE). However, most transcribed chromatin is associated with the nuclear matrix (chromatin fraction PE), which is insoluble (1998 report, Fig. 4). To overcome this obstacle, an alternate procedure using the ChIPs technology was developed to isolate transcriptionally active chromatin associated with highly acetylated histones.

ii. Dynamics of Histone Acetylation in Human Breast Cancer Cells

Transcribed chromatin is associated with highly acetylated histones [15]. The dynamic acetylation of lysines in the N-terminal tails of histones plays an important role in transcription. The ChIPs technology with anti-acetylated histone antibodies is designed to immunoprecipitate transcriptionally active chromatin that bears highly acetylated histones. Before embarking with the ChIPs procedure, it was important to understand the dynamics of histone acetylation in breast cancer cells and the effect that estradiol had on this process. At the time that we initiated these studies, literature searches revealed that knowledge of histone acetylation dynamics in human breast cancer cells was very limited, with only one report (1978) being published [9]. In this study, the cell culture, ligand treatment, pulse-chase labeling, electrophoresis conditions were not optimized for studying the effect of estradiol on histone acetylation. Since we believed that it was important to understand the effect of estradiol on histone acetylation

dynamics in breast cancer cells, in the last two years we have performed an in-depth study to obtain this information.

a. Estradiol increases histone acetylation in ER positive T47D5 cells, but does not affect histone acetylation in ER negative MDA MB 231 cells

To study the effect of estradiol on histone acetylation in ER positive breast cancer cells, pulse-chase labeling studies with T47D5 cells were performed. The percentage of the hyperacetylated histone isoforms was calculated by scanning the bands in fluorographic films. The experiment showed that estradiol increased histone acetylation in ER positive breast cancer cells (1999 annual report, Fig.2).

To confirm the affect of estradiol on histone acetylation, Western blot analysis using anti-hyperacetylated H4 antibodies was performed. T47D5 cells were grown in estrogen deplete medium for 3 days. To inhibit new protein synthesis, cells were incubated in the presence of cycloheximide (10 μ g/ml) 30 minutes before labeling. Cells were incubated with 10 nM estradiol or ethanol (vehicle) for 20 minutes at 37° C. The histones were extracted, and electrophoresed on a SDS 15% polyacrylamide gel or AUT 15% polyacrylamide gel. Anti-acetyl H4 antibodies were used in the Western blot analysis. Fig. 1, panel B shows the histones resolved by SDS PAGE. The level of acetylated H4 was greater in E2 treated cells compared to the H4 of cells grown without E2. To further analyze the acetylated histone isoforms, a high resolution AUT electrophoretic system was applied. AUT PAGE resolves histones according to molecular size, positive charges and hydrophobicity. In panel A (Fig.1), Western blot analysis shows that the levels of di- and tri-acetyl H4 isoforms increased, with tri-acetyl H4 increasing more significantly in E2 treated cells. For cells incubated with butyrate, a histone deacetylase inhibitor, the acetylated H4 isoforms increased. These results show that as soon as twenty minutes after estradiol was added to the cells that there was an increase in the steady state level of acetylated H4 in human breast cancer cells.

To decide if ER had a role in the observed effect of estradiol on histone acetylation status, we repeated this analysis with ER negative MDA MB 231 breast cancer cells. The conditions for labeling acetylated histones of MDA MB 231 cells were the same as those used with T47D5 breast cancer cells (1999 annual report, Methods).

MDA MB 231 cells, grown under estrogen deplete or replete (10 nM E2) conditions, were labeled with [^3H]-acetate at 37°C for 20 minutes. The Coomassie Blue stained gel and fluorogram of labeled histones isolated from the MDA MB 231 cells are shown in Fig. 2. After scanning the fluorographic film, the percentage of histone H4 that was hyperacetylated was calculated. The ratio of hyperacetylated isoforms (tri- and tetra-acetyl) H4 in the total H4 in E2 treated MDA MB 231 cells was approximately 17.5%, which was very similar to the observed 18% for cells without E2 treatment. In last year's report, we found that estradiol increased the percentage of hyperacetylated H4 to approximately 12.5% from 9% for H4 from T47D5 cells incubated without E2 (1999 annual report, p11). This result demonstrated that estradiol does not affect histone acetylation in ER negative, hormone independent breast cancer cells. The pulse-chase labeling of ER negative breast cancer cells will be repeated, and the Western blot analysis of histones from ER negative cells treated with estradiol and butyrate will be done.

b. Estradiol does not affect the rate of histone acetylation; however it affects the rate of histone deacetylation in T47D5 cells

Finding that estradiol increased the level of acetylated histones, we determined the rates of histone acetylation and deacetylation and the influence of estradiol on these rates in T47D5 cells. The cells were pulse-chase labeled with [^3H]-acetate (1999 annual report, Fig. 3), and the rate of acetylation was calculated. Two rates of acetylation for histones H4, H3 and H2B ($t_{1/2} = 8'-10'$ and $t_{1/2} = 200'-350'$) were observed in T47D5 cells (1999 report, Fig. 5).

To understand the effect of estradiol on the rate of histone acetylation, T47D5 cells were pulse-chase [^3H]-acetate labeled in the presence or absence of 10 nM estradiol. The rates of histone H4 in the presence or absence of estradiol were very similar (rapid rate with $t_{1/2} = 8'$ and slow rate with $t_{1/2} = 200'-350'$) (1999 report, Fig. 6 and 7). This result demonstrated that estradiol did not affect the rate of histone acetylation.

Does estradiol affect the rate of histone deacetylation? The rates of histone deacetylation in T47D5 cells treated with or without E2 were determined. T47D5 cells were grown in the estrogen deplete medium for 3 days. The protein synthesis inhibitor,

cycloheximide, was added 30 minutes before pulse-chase labeling. The cells were incubated with 10 mM butyrate in the presence of 10 nM estradiol or ethanol (vehicle) for two hours. After washing to remove butyrate, the cells were grown in the fresh medium with or without E2. The histones extracted from the cells were resolved by AUT PAGE, and analyzed by fluorography. The rates of histone deacetylation were calculated (1999 annual report, Fig. 9). Importantly, in E2 treated cells, the rate of deacetylation of H4-Ac4 was slower than that of H4-Ac4 in cells grown without E2 ($t_{1/2}=8'-10'$ versus $t_{1/2}=6'$). These observations demonstrated that estradiol increases the steady state of H4 acetylation in T47D5 cells by reducing the rate of histone deacetylation.

c. Estradiol decreases the amount of ER, but not HDAC1, in T47D5 cells

A question arises: Is a change in the level of HDAC responsible for the change in the rate of histone deacetylation? The levels of ER and HDAC1 in cells incubated with or without E2 for different times were analyzed by Western blotting. The conditions for treating the cells were the same as those used in the experiment that determined the effect of E2 on the rate of histone deacetylation (1999 annual report, Method iii, p. 7). Two groups of T47D5 cells were grown in estrogen deplete conditions for 3 days. The protein synthesis inhibitor cycloheximide (10 $\mu\text{g/ml}$) was present 30 minutes before labeling (Fig. 3, panel A). One group of cells was incubated with 10 nM E2 in the presence of 10 mM butyrate, and another group of cells was incubated with vehicle (ethanol) in the presence of 10 mM butyrate at 37°C for 2 hours. After washing to remove the butyrate, the cells were incubated in fresh medium replaced with or without E2 for 0 to 240 minutes. The cells were lysed in SDS lysis buffer. Anti-ER and anti-HDAC1 antibodies were used to immunochemically stain the membranes (Fig. 3, panel B). After a 4-hour incubation with E2 (equal to 120' in Fig. 3 panel B), ER levels declined. However the HDAC1 levels did not change. It should be noted that the 0' time after removing butyrate corresponds to 2 hours treatment of E2 and 2.5 hours incubation with cycloheximide (Fig. 3, panel A). In the T47D5 cell [3H]-acetate labeling experiment (1999 annual report, Fig. 2), the acetyl-H4 isoforms were increased after 20 minutes E2 treatment; these cells would have also been incubated for 50 minutes with

cycloheximide. Our results show that after 20 minutes estradiol did not affect the levels of ER and HDAC1. Thus, decreased rate of histone deacetylation in estradiol treated cells was not a consequence of reduced HDAC1 levels.

d. Estradiol does not affect the HDAC activity in T47D5 cells

To understand the mechanism by which estradiol affects the rate of histone deacetylation, HDAC activity of HDAC in T47D5 cells incubated with or without estradiol were studied. The cells were grown in the estrogen deplete medium for 3 days, and incubated in the presence or absence of 10 nM estradiol for 20 minutes. The same numbers of cells treated with or without E2 were lysed in TNM buffer containing 0.25% NP-40 and passed through a syringe with a #22 gauge needle. Fig. 4, panel B shows that the HDAC activity in the lysed cells treated with E2 was the same as that of lysed cells incubated without E2. The levels of HDAC1 and HDAC2 in cell lysate were determined in Western blot experiment. Fractions from an equal number of cells were resolved by SDS PAGE, and the proteins were transferred onto nitrocellulose membrane. Western blot analysis (Fig. 4A) shows that the levels of HDAC1 and HDAC2 in the cell lysates from cells treated with or without E2 were not significantly different. These experiments showed that estradiol did not affect the activity and level.

e. ER-HDAC1, Sp1-HDAC1 and Sp3-HDAC1 complexes in T47D5 cells

We have shown previously that ER when bound to hydroxytamoxifen recruits the HDAC complex containing HDAC 1 and 2 [16]. However, it is not known whether unliganded ER recruits the HDAC complex. It is conceivable that estradiol affects ER capability to recruit the HDAC complex. Immunoprecipitation experiments were performed to analyze these complexes. First, the efficiency of anti-ER and anti-HDAC1 antibodies to immunoprecipitate their respective proteins was tested. T47D5 cells were lysed in IP buffer and sonicated. After centrifugation, the supernatant was used as the cell lysate for IP. Both ER and HDAC1 are associated with the nuclear matrix. To check solubilization efficiency, anti-ER and anti-HDAC1 antibodies were used to probe Western blot membranes of proteins from the supernatant (cell lysate) and pellet. The Western blot analysis shown in Fig. 5 panel A demonstrated that most HDAC1 and ER

were in the cell lysate fraction. Fig. 5 panel B shows the HDAC activity in the cell lysate and pellet fractions. Most HDAC activity was in the cell lysate fraction. Second, the efficiency of the IP conditions was tested. Two units of A_{260} of the cell lysate were incubated with 2 μ g of anti-HDAC1 antibody. The activity and the level of HDAC1 in the bound (IP) and unbound fractions were analyzed. Western blot analysis (Fig. 6, panel A) shows that approximately 99% of HDAC1 and 90% of ER were in the IP fraction. HDAC activity in the IP fraction precipitated by the anti-HDAC1 antibody was tested. The results showed that HDAC1 contributed approximately 50% of the total cellular HDAC activity (Fig. 6, panel B).

To determine if HDAC1 was associated with ER, the immunoprecipitates were analyzed by Western blotting (Fig. 6, panel A). The immunoprecipitate obtained with anti-ER antibodies was associated with HDAC1. Western blot analysis of the anti-HDAC1 immunoprecipitate showed, however, that most ER was not associated with HDAC1. It has been shown previously that HDAC1 is associated with Sp1 [17]. As a control, we analyzed immunoprecipitates isolated with anti-Sp1 antibodies (Fig. 7, panel A). Western blot analysis shows that HDAC1 was associated in a complex with Sp1 in T47D5 cells (Fig. 7, panel A). To test whether estradiol affected the association of HDAC1 with these complexes, the activities and presence of HDAC in the complexes were analyzed. Sp1 and ER complexes from cell lysates from the cells incubated with or without E2 were immunoprecipitated with anti-Sp1 and anti-ER antibodies. There are suggestions in the literature that Sp3 also interacts with HDAC1 [18], but an interaction between Sp3 and HDAC1 has not yet been demonstrated. Thus, we also analyzed complexes immunoprecipitated with anti-Sp3 antibodies. Regardless of whether the cells were grown under estrogen replete or deplete conditions, ER, Sp1 and Sp3 complexes were associated with HDAC activity (Fig. 7, panel B). However, the level of HDAC activity associated with the Sp3 complex was greater than that of the Sp1 and ER complexes and far less than that of the complexes immunoprecipitated with anti-HDAC1 antibodies. The results also showed that estradiol did not alter the activity of HDAC associated with Sp1. However, a reproducible decline in HDAC activity was observed for ER complexes, while a slight increase in activity was seen with Sp3 complexes isolated from estradiol treated cells. Western blot experiments showed that

HDAC1 was associated with Sp1 and ER of estradiol treated cells (Fig. 7, panel A). Although estradiol treatment reduced the HDAC activity associated with ER, we believe that the low level of HDAC activity associated with ER is insufficient to account for the decreased rate of histone deacetylation.

f. Estradiol affects the sub-cellular distribution of ER, but not HDAC in T47D5 cells

Our studies presented above demonstrated that the estradiol-induced reduction in histone deacetylation rates was not a consequence of an alteration in HDAC activity. We tested the idea that estradiol altered the sub-cellular distribution of HDAC. If a sub-cellular redistribution of HDAC occurred, the HDAC may locate to a region where it is not enzymatically active. To do these analyses, we developed a novel protocol to fractionate cells (Fig. 8). Cells were lysed in TNM buffer without any detergents to minimize loosely bound nuclear proteins from leaking out of the nuclei. The cells were lysed by passing cells through a syringe with a #22 gauge needle. The cytosol and nuclei were separated by centrifugation. The integrity of the nuclei was inspected under a microscope. The nuclei were resuspended in TNM buffer with 0.5% Triton X-100, and incubated on ice for 5 minutes to release loosely bound nuclear proteins (Triton-S). The resulting pellet contained the tightly bound nuclear proteins, which includes proteins associated with the nuclear matrix fraction (Triton-P).

The distribution of ER in the cell fractions from the cells treated with or without E2 for 20 minutes was determined by Western blot analyses. The cellular fractions, equal to same number of cells, were loaded onto a SDS polyacrylamide gel, then subsequently transferred onto a nitrocellulose membrane. The membrane was immunochemically stained with an antibody against human ER α . ER levels in the cell lysate untreated or treated for 20 minutes with 10 nM E2 did not change (Fig. 9, compare lane 1 with lane 6). Most ER was in the nuclei fraction. However, after E2 treatment for 20 minutes, there was a marked decline in the level of ER leaking from the nuclei into the cytosol fraction (Fig. 9, compare lane 2 with lane 7). Fractionation of the nuclear proteins into those that were loosely bound from those that were tightly bound revealed also a shift in the sub-cellular partitioning of ER. Comparison of the ER levels

in the Triton-S and Triton-P fractions from cells treated with or without E2 showed that 20 minutes of estradiol treatment caused ER to go from a loosely bound nuclear form to a tightly bound nuclear form (Fig. 9). This observation is agreement with reports from our group and Dr. Mancini's group [19,20]. Dr. Mancini's group and we have shown that the tightly bound form of ER is associated with the nuclear matrix.

There are two classes of HDACs in mammalian cells. HDAC1, HDAC2, HDAC3 and HDAC8 belong to class I. HDAC4, HDAC5, HDAC6 and HDAC7 belong to class II [15]. The distribution of class I of HDAC among the cellular fractions was determined by Western blot analyses using antibodies against human HDAC1, HDAC2 and HDAC3. The analyses showed that the levels of HDAC1 and HDAC2 in the cell lysate treated with or without E2 were similar (Fig. 9, compare lane 1 with lane 6). In the cells incubated with or without E2, most HDAC1 and HDAC2 was found in the nuclei (Fig. 9, compare lane 2 with lane 3 and lane 7 with lane 8). Triton X-100 released only a small amount of HDAC1 and HDAC2 from the nuclei (Fig. 9, compare lane 4 with lane 5 and lane 9 with lane 10). These results suggested that most HDAC1 and HDAC2 are associated with the nuclear matrix. HDAC3 was also in the nuclei fraction, but only approximately half of HDAC3 was associated with the tightly bound nuclear protein fraction. This result agrees with the findings that HDAC 1 and 2 exist in different complexes than those that contain HDAC3 [13]. In contrast to the class I HDACs, HDAC4 was found exclusively in the loosely bound nuclear protein fraction (Fig. 9 lanes 4 and 9). Importantly, the Western blot analyses demonstrated that estradiol did not induce a subnuclear relocalization of these HDACs such as that observed with ER. We conclude that estradiol does not affect the subnuclear trafficking of class I and class II HDACs in T47D5 human breast cancer cells.

HDAC activities of cell lysate, cytosol, nuclei and nuclear fractions, Triton-S and Triton-P, were analyzed (Fig. 10). The majority of the nuclear HDAC activity was present in the tightly bound nuclear fraction (Fig. 10, panel B). This result in agreement with our previous studies that showed that HDAC was a nuclear matrix protein [21,22]. The percentage of HDAC activities of Triton-S fraction from cells treated with E2 was similar to the activity from cells incubated without E2 (the difference is $\pm 0.5\%$ ($n=3$)).

HDAC activities of cell lysate, cytosol and nuclei from the cells treated with or without E2 were also similar (Fig. 10, panel A).

The results of the HDAC protein distribution and HDAC activity appeared to disagree (compare Fig. 9 with 10). The cytosol had high HDAC activity, while the Western blot analyses showed that none of the HDACs partitioned preferentially with the cytosol fraction. An explanation for this discrepancy is as follows. The cytosol fraction is devoid of chromatin (that is, nucleosomal histones), while the nuclear fractions contain chromatin. Thus, the specific activity of the substrate (labeled histones) is much greater for the cytosol fraction than it is for the nuclear fraction. We conclude that it is not appropriate to decide the sub-cellular partitioning of HDACs by assaying fractions that have different amounts of nucleosomal histones.

g. Estradiol functions on the redistribution HAT activity and ER associated cofactors in T47D5 cells

From the previous experiments, we learned that estradiol alters the subnuclear trafficking of ER, but not that of the HDACs. We next tested the idea that estradiol would affect the sub-cellular distribution of HATs. HAT activity assays and Western blot analyses were performed. The cell lysate, cytosol, nuclei, and loosely bound and tightly bound nuclear fractions were prepared as previously described. The cell fractions, equal to the same number of cells, were assayed for HAT activity. Fig. 11 shows the cellular distribution of HAT activity. HAT activities in the lysed cell fractions from cells treated with or without E2 did not significantly change. However, the HAT activity of nuclei from cells incubated with E2 was greater than that of nuclei from cells grown under estradiol deplete conditions (Fig. 11, panel A). Fractionation of the nuclear fraction showed both the loosely bound and tightly bound nuclear proteins had HAT activity. However, the tightly bound nuclear proteins demonstrated a differential HAT activity with preparations isolated from cells grown with and without estradiol. After twenty minutes of estradiol treatment, we observed an increase in the HAT activity associated with the Triton-P, tightly bound nuclear protein fraction (Fig. 11, panel B). These results are consistent with the idea that HAT is associated with the nuclear matrix [10] and that the subnuclear

trafficking of HATs is affected by estradiol in hormone responsive breast cancer cells [20].

In mammalian cells, many transcriptional coactivators contain HAT activity [13,23]. Steroid receptor cofactor 1 (SRC-1) and SRC-3 (AIB1/RAC3/ACTR) are HATs. In a ligand dependent manner, SRC-1 and SRC-3 associate with ER. The effect of estradiol on the subnuclear trafficking of SRC-1 and SRC-3 was determined in Western blot experiments with antibodies against SRC-1 and SRC-3. Fig. 12 shows that most SRC-1 was in the nuclei (Fig. 12, compare lane 2 with lane 3, and lane 7 with lane 8). Triton X-100 released less SRC-1 from nuclei in cells treated with E2 than from nuclei of cells treated without E2 (Fig. 12, compare lane 4 with lane 5, and lane 9 with lane 10). The same trend was observed with SRC-3. This result demonstrated that estradiol caused an alteration in the subnuclear trafficking of SRC-1 and SRC-3 with SRC-1 and SRC-3 becoming more tightly bound in the nucleus following twenty minutes of estradiol treatment. This tightly bound state is probably nuclear matrix associated SRC-1 and SRC-3. Our observations with SRC-1 agree with the results of Dr. Mancini and colleagues who used a bioluminescent derivative of SRC-1 to study the effect of estradiol on subnuclear trafficking of this coactivator [20].

In addition to SRC-1 and SRC-3, we determined if estradiol had an effect on the subnuclear distribution of the HATs CBP, PCAF-A and PCAF-B. Western blot analyses of the cellular fractions shown in Fig. 13 demonstrated that in contrast to SRC-1 and SRC-3, estradiol did not affect the partitioning of these HATs. It is interesting to note that most of CBP, PCAF-A and PCAF-B were present in the tightly bound nuclear protein fraction, suggesting that these transcriptional coactivators / HATs are predominantly associated with the nuclear matrix.

To summarize our findings, we observed an increase in histone acetylation as soon as twenty minutes after the addition of estradiol to hormone dependent breast cancer cells. In contrast, the level of acetylated histones of hormone independent breast cancer cells was not affected by estradiol. Analyses of histone acetylation and histone deacetylation rates showed that the rate of histone acetylation was not affected in estradiol treated hormone dependent cells. However, histone deacetylation rates declined following estradiol administration. Analyses of HDAC activities, levels and

cellular distribution showed that estradiol did not affect on any of these parameters. Estradiol, however, had a profound effect on the subnuclear trafficking of ER. When bound to estradiol, ER will recruit SRC-1 and SRC-3 to estrogen responsive genes. Accordingly, estradiol affected the subnuclear trafficking of SRC-1 and SRC-3, ER-associated coactivators. We propose that SRC-1 and SRC-3 are recruited away from sites that have both these HATs and HDACs (Fig. 14). The removal of these HATs from specific chromatin sites would result in an absence of histone acetylation at these sites, leaving the HDACs without a substrate. This could explain why histone deacetylation rates declined. Further, at hormone responsive gene promoters, the balance of HATs and HDACs would swing in favor of histone acetylation once estradiol was added to the cells. In T47D5 cells it has been experimentally determined that there are 1.86×10^5 ER molecules per cell [24]. Thus, the estradiol induced alterations in the subnuclear trafficking of ER and associated HATs may be sufficient to account for the increased rates of histone acetylation that we observe in these cells.

h. Chromatin immunoprecipitation to isolate the transcriptionally active DNA

Based upon the observations that hyperacetylated histones are associated with transcriptionally active nucleosomes, the ChIPs protocol with anti-acetylated histone H3 or H4 antibodies was performed (Fig. 15). The optimum condition for ChIPs protocol was tested in studies of last year. We believe that the quality and the specificity of antibodies are important factors for successful application of the ChIPs technique. We have prepared the antibodies against N-terminal peptides with acetylated lysine (9,14,18,23) of H3 and acetylated lysine (5,8,12,16) of H4. Only one batch of antiserum against acetylated H3 worked with lower background (not shown). Unfortunately, the rabbit did not survive after a few injections of the antigen. The antibodies against acetylated H3 and acetylated H4 are available commercially. In our previous report we showed the specificity of anti-acetylated H3 antibodies (Upstate) (1999 annual report, p17).

To test the suitability of anti-acetylated H3 and anti-acetylated H4 antibodies to isolate transcriptionally active DNA, we applied the ChIPs protocol to fractionate DNA from ER positive, hormone dependent breast cancer cells (T47D5) and ER negative,

hormone independent breast cancer cells (MDA MB 231). Two units of A₂₆₀ cell lysate were incubated with 2 µg of anti-acetylated H3 or anti-acetylated H4 antibodies. The yield of DNA isolated by ChIPs with either antibody was low, with the average yield being approximately 0.4 to 0.8 µg per A₂₆₀ of cell lysate. The input DNA and DNA isolated by ChIPs were amplified by PCR with specific primer sets and analyzed on agarose gels. The primer sets chosen monitored the human ER α exon I, *c-myc* exon I, and GAPDH exon VII. Human ER α gene is transcribed in T47D5, but not MDA MB 231 cells. Human *c-myc* and GAPDH, a housekeeping gene, are transcribed in both cell lines. Analyses of the input DNA showed that the *c-myc*, GAPDH and ER sequences were present, with the exception that the ER PCR product was not detected in the input DNA from MDA MB 231 cells (Fig. 16). In a repeat of this experiment with anti-acetylated H4 antibodies the ER PCR fragment was detected in the input DNA of MDA MB 231 cells (Fig. 17).

Fig. 16 and 17 show that the 279-bp long *c-myc* exon I and 284-bp housekeeping GAPDH gene exon VII DNA fragments were found in DNA isolated by ChIPs from T47D5 and MDA MB 231 cells. However, the 171-bp ER exon I fragment was detected only in the immunoprecipitate from T47D5 cells. This result provides support for the plan to use the ChIPs protocol with antibodies to acetylated histones to isolate expressed DNA.

A recent publication demonstrated that histone H4 acetylation was more prominent than H3 acetylation on hormone responsive promoters in human breast cancer cells [14]. This result suggested that antibodies to acetylated H4 might be superior to antibodies against acetylated H3 in isolating transcriptionally active chromatin. However, this study [14] did not analyze the coding region of hormone responsive gene where the acetylation status of the nucleosomal histones may be quite different from that at the promoter [13]. Fig. 16 shows that the intensity of *c-myc* fragments amplified from DNA isolated with anti-acetylated H4 antibodies was slightly greater than that of the fragments amplified from DNA isolated by anti-acetylated H3 antibodies for both the T47D5 and MDA MB 231 cells. The abundance of the ER PCR fragment was marginally greater in the immunoprecipitate with anti-acetylated H4 antibodies. No difference was observed with the intensities of the GAPDH PCR

fragments. These results show that highly acetylated H3 and H4 are associated with the coding regions of the *c-myc* and GAPDH genes in T47D5 and MDA MB 231 cells. Importantly, the results show that the coding region of ER in T47D5, but not MDA MB 231 cells, is associated with highly acetylated H3 and H4 histones. Thus, the marked differential acetylation of H3 and H4 observed at the promoter of estrogen responsive genes is not observed along the coding regions.

Following the isolation of transcriptionally active DNA, we had planned to apply a PCR-Select Subtract protocol to isolate genes differentially expressed in T47D5 and MDA MB 231 cells. This project will be continued with funds from the Canadian Institute of Health.

D. RECOMMENDATIONS

In the three years' study, we have optimized the procedure to isolate transcriptionally chromatin from human breast cancer cells. This protocol should isolate "all" of transcriptionally active DNA, including genes that code for strictly nuclear RNA. Approximately 70% of RNA remain in the nuclei and may be tightly associated with nuclear matrix, contributing to the structure of the nuclear matrix. In the first year's study, organomercury column and Sulfolink Coupling gel affinity column chromatography was applied to isolate transcribing chromatin. We found that in human breast cancer cells most transcriptionally active chromatin was associated with nuclear matrix, which is insoluble. Thus, the column chromatography approach had serious limitations in isolating transcriptionally active chromatin. To resolve this problem we applied the ChIPs protocol to isolate transcriptionally active chromatin from cell lysates. Our results show that the ChIPs protocol with antibodies to acetylated H3 and H4 histones is an effective method to isolate transcriptionally active DNA. The application of the PCR-Select Subtract protocol with the ChIPs DNA should be able to isolate differentially expressed DNA from human breast cancer cells.

We demonstrated that estradiol rapidly elevates the steady state level of histone acetylation in ER positive, hormone responsive breast cancer cells. Estradiol has an immediate effect on the subnuclear trafficking of ER and ER-associated transcriptional coactivators with HAT activity. We proposed that this alteration in subnuclear trafficking

accounts for the increased level of histone acetylation (Fig. 14). Although our results did not provide evidence for changes in the subnuclear trafficking of HDACs, it is possible that these enzymes are moving to different sites on the nuclear matrix. Our biochemical approach would not detect this. Immunolocalization analysis of the nuclear matrices with antibodies to the HDACs by confocal microscopy would be one method to test this idea. Our model shown in Fig. 14 suggests that some regions of breast cancer chromatin will become deacetylated, while the chromatin of hormone responsive genes will become highly acetylated following the addition of estradiol. The ChIPs protocol would be an ideal method to test this model.

6. KEY RESEARCH ACCOMPLISHMENTS

- Organomercury column and Sulfolink Coupling gel column enriched for transcriptionally active chromatin from salt-soluble chromatin fractions (S0 and SE).
- Dynamics of histone acetylation in human breast cancer cells:
 - ER+, hormone dependent (T47D5) and ER-, hormone independent (MDA MB 231) breast cancer cells have two rates of histone acetylation.
 - Approximately 10% of the histones is rapidly hyperacetylated ($t_{1/2} = 6'-10''$ for monoacetylated H4, H3 and H2B).
 - The bulk of the core histones is slowly acetylated ($t_{1/2} = 200'-300'$ for monoacetylated H4).
 - Rapidly hyperacetylated H4 is rapidly deacetylated ($t_{1/2} = 6'$ for tetraacetylated H4).
 - Estradiol increases histone acetylation in T47D5 (ER+) cells, but not in MDA MB 231 (ER-) cells.
 - Estradiol affects the rate of histone deacetylation, but not the rate of histone acetylation in T47D5 cells.
- The level of ER, but not HDAC1, is reduced in T47D5 cells treated with estradiol for four hours.
- Estradiol does not affect HDAC activity and the level of HDAC in T47D5 cells.
- Estradiol does not affect the subnuclear distribution of HDAC1, 2, 3 and 4 in T47D5 cells treated for 20 minutes.

- HDAC1, HDAC2, about 50% of HDAC3, and none of HDAC4 are associated with the nuclear matrix of T47D5 cells.
- Estradiol administration to T47D5 results in a rapid movement of ER to the nuclear matrix compartment.
- Estradiol affects the subnuclear trafficking of ER associated transcriptional coactivators (SRC-1 and SRC-3) in 20 minute treated T47D5 cells.
- Estradiol increases the HAT activity associated with the nuclear matrix of T47D5 treated for 20 minutes.
- HDAC1 is associated with ER and Sp1 complexes in T47D5 cells.
- Estradiol slightly reduces HDAC activity of ER complexes in 20 minute treated T47D5 cells.
- The ChIPs protocol with anti-acetylated H4 antibodies and/or anti-acetylated H3 antibodies is an effective method to isolate transcriptionally active DNA from human breast cancer cells.

7. REPORTABLE OUTCOMES

A. MANUSCRIPTS, ABSTRACTS, PRESENTATIONS

Spencer VA, Coutts AS, Samuel SK, Murphy LC, Davie JR: Estrogen regulates the association of intermediate filament proteins with nuclear DNA in human breast cancer cells. *J.Biol.Chem.* 273:29093-29097, 1998

Davie JR, Samuel SK, Spencer VA, Holth LT, Chadee DN, Peltier CP, Sun J-M, Chen HY, Wright JA: Organization of chromatin in cancer cells: role of signalling pathways. *Biochem.Cell Biol.* 77:265-275, 1999

Davie JR, Spencer VA: Control of histone modifications. *J.Cell Biochem.* 32/33:141-148, 1999

Spencer VA, Davie JR: Role of covalent modifications of histones in regulating gene expression. *Gene* 240:1-12, 1999

Davie JR, Spencer VA: Signal transduction pathways and the modification of chromatin structure. *Prog. Nucl. Acids Res. Mol. Biol.* 65: 299-340, 2000

Davie JR, Moniwa M: Control of chromatin remodeling. Crit Rev.Eukaryot.Gene Expr. in press, 2000

Histone acetylation in human breast cancer cells. The Department of Defense Breast Cancer Research Meeting, Atlanta, page 133, 2000

Sun J-M, Chen H-Y, Davie JR: Estradiol rapidly reduces the rate of histone deacetylation in human breast cancer cells. Histone Deacetylase Chromatin Remodeling Workshop, NCI, NIH, Washington, DC, 2000 – Presentation

Sun J-M, Chen H-Y, Davie JR: Dynamic histone acetylation in human breast cancer cells. West Coast Chromatin and Chromosomes Meeting, Asilomar, CA, December, 2000 - Presentation

B. EMPLOYMENT OR RESEARCH OPPORTUNITIES APPLIED FOR AND/OR RECEIVED BASED ON EXPERIENCE/TRAINING SUPPORTED BY THIS AWARD

Virginia Spencer, a Ph.D. candidate, supported by this award was successful in obtaining a studentship from Manitoba Health Research Council, Cancer Research Society, Inc. and the prestigious National Cancer Institute of Canada. Virginia decided to take the National Cancer Institute of Canada studentship award.

The following is a list of personnel receiving pay from the research effort.

Jian-Min Sun, research associate

Virginia Spencer, graduate student

Katherine Dunn, graduate student

Mariko Moniwa, graduate student

Jason Neufeld, technician

Jeannette LeBlanc, technician

Helen and Charlene Bergen, dishwasher

8. CONCLUSIONS

There is an intimate relationship between transcription and histone acetylation. Enzymes catalyzing histone acetylation, histone acetyltransferases and histone deacetylases, are transcriptional coactivators and repressors, respectively [4,15,25]. Histone acetylation of a gene locus is initiated by the recruitment of a histone acetyltransferase/coactivator by transcription factors bound to the gene's promoter or enhancer [1,26].

Estrogens and antiestrogens regulate the transcription of target genes in hormone dependent breast cancer cells by binding to a ligand-dependent transcription factor, the estrogen receptor (ER). Coactivators and corepressors interact with ER in a ligand dependent manner. ER coactivators often have histone acetyltransferase (HAT) activity, while ER corepressors often have histone deacetylase (HDAC) activity [25,27,28]. Thus, ER can recruit multiprotein complexes containing either HAT or HDAC activities. It has been proposed that transcription activators (e.g., ER-estradiol) recruit HAT complexes, which in turn acetylate histones, forming a transcriptionally permissive chromatin structure [1,29]. Alternatively, repressors (e.g., ER-hydroxytamoxifen, which we have shown binds to the SinAP30 component of the HDAC complex) recruit HDAC complexes which in turn deacetylate histones, forming a repressive chromatin structure [1,16]. These models suggest that control of histone acetylation has a central role in hormone signaling.

We have determined the kinetics of histone acetylation in hormone dependent and hormone independent breast cancer cells. One population of core histones is characterized by rapid hyperacetylation ($t_{1/2} = 8$ minutes for monoacetylated H4) and

rapid deacetylation ($t_{1/2}$ = 6 minutes). Only a small percentage (10%) of the histones is involved in this rapidly reversible and extensive acetylation. These dynamically acetylated core histones are thought to be bound principally to transcriptionally active DNA [21,30,31]. Another population of histones, which includes the bulk of the core histones, is slowly acetylated with $t_{1/2}$ = 150 to 300 minutes for monoacetylated H4.

We made the novel observation that estradiol increases the steady state level of histone acetylation in hormone dependent T47D5 breast cancer cells. Estradiol increased the steady state level of histone acetylation by decreasing the rate of histone deacetylation. The rapid rate of histone acetylation was not affected by estradiol. We proposed that the addition of estradiol to T47D5 cells grown under estrogen deplete conditions alters the balance of histone acetyltransferases and histone deacetylases at transcriptionally active chromatin sites by preventing the recruitment of histone deacetylases.

Analyses of HDAC activities, levels and cellular distribution showed that estradiol did not affect these parameters. Estradiol, however, had a profound effect on the subnuclear trafficking of ER. Once bound to estradiol, ER recruits SRC-1 and SRC-3 to estrogen responsive genes. Consistent with this model, estradiol affected the subnuclear trafficking of SRC-1 and SRC-3, ER-associated coactivators. We propose that SRC-1 and SRC-3 are recruited away from sites that had both these HATs and HDACs. The removal of these HATs from specific chromatin sites would result in an absence of histone acetylation at these sites, leaving the HDACs without a substrate. This could explain why histone deacetylation rates declined in estradiol treated cells. Further, at hormone responsive gene promoters, the balance of HATs and HDACs

would swing in favor of histone acetylation once estradiol was added to the cells. In T47D5 cells it has been experimentally determined that there are 1.86×10^5 ER molecules per cell [24]. Thus, the estradiol induced alterations in the subnuclear trafficking of ER and associated HATs may be sufficient to account for the increased rates of histone acetylation that we observe in these cells.

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10. APPENDICES**A. ACRONYM AND SYMBOL DEFINITION**

ER	estrogen receptor
EDTA	[ethylenebis(oxyethylenenitrio)]tetraacetic acid
PCR	polymerase chain reaction
FBS	fetal bovine serum
SDS	sodium dodecyl sulphate
AUT PAGE	Acetic acid-Urea-Triton X-100 polyacrylamide gel
GAPDH	glyceradehyde 3-phosphate dehydrogenase
E2	estradiol
Ac3	tri-acetylated histone isoform
Ac4	tetra-acetylated histone isoform
ChIPs	chromatin immunoprecipitation
HAT	histone acetyltransferase
HDAC	histone deacetylase
DMEM	Dulbecco's modified Eagle medium
PBS	phosphate buffered saline
PCAF	p300/CBP associated factor
CBP	CREB-binding protein
PMSF	phenylmethylsufonyl fluoride
S0	soluble chromatin fraction after nuclease digestion
SE	10 mM EDTA soluble chromatin fraction
PE	10 mM EDTA insoluble chromatin fraction
SRC-1	steroid receptor cofactor 1
SRC-3	steroid receptor cofactor 3, also named AIB1, RAC3 and ACTR
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
Triton-S	Triton X-100 soluble nuclear fraction
Triton-P	Triton X-100 insoluble nuclear fraction
IP	immunoprecipitate
PAGE	polyacrylamide gel electrophoresis

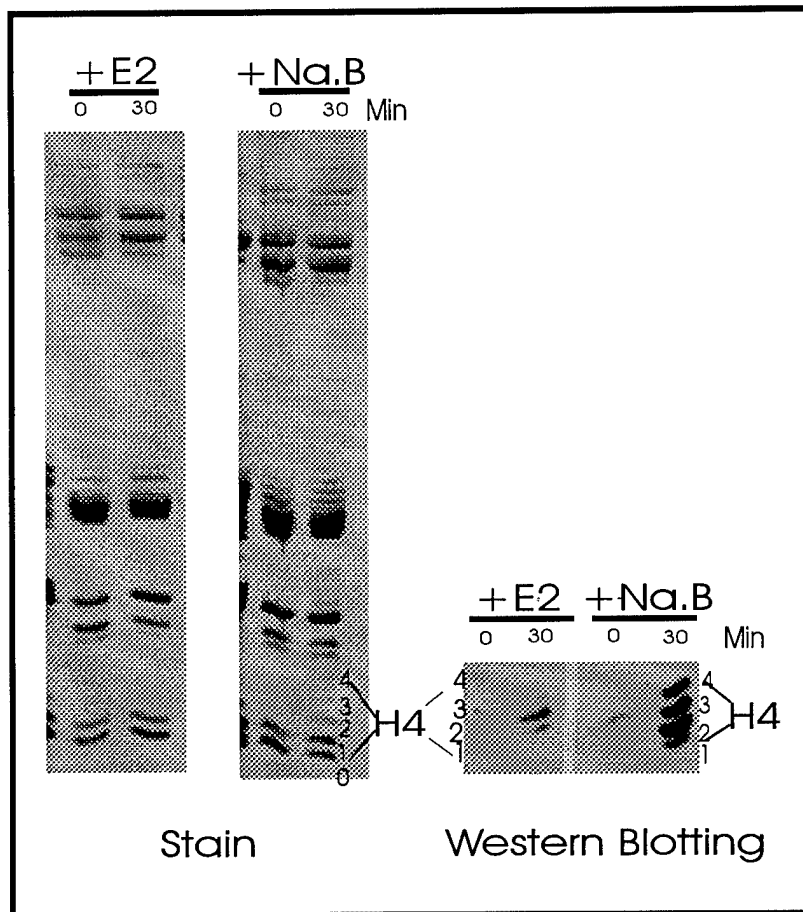
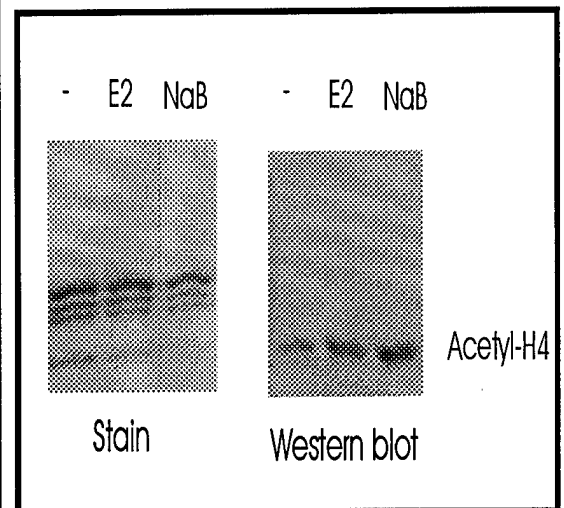
B. FIGURES**A****B**

Fig. 1. Western blot analysis of the affect of estradiol on histone acetylation in T47D5 cells, which are ER positive and hormone dependent. The cells were grown in phenol red free DMEM medium containing 7% charcoal stripped FBS for three days, incubated in the presence or absence of 10 nM estradiol (E2) with or without 10 mM sodium butyrate (Na.B) for 20 minutes. The histones were resolved by AUT 15% PAGE (Panel A) and SDS 15% PAGE (Panel B), subsequently stained with Coomassie blue or transferred to nitrocellulose membranes and immunochemically stained with anti-acetylated H4 antibodies.

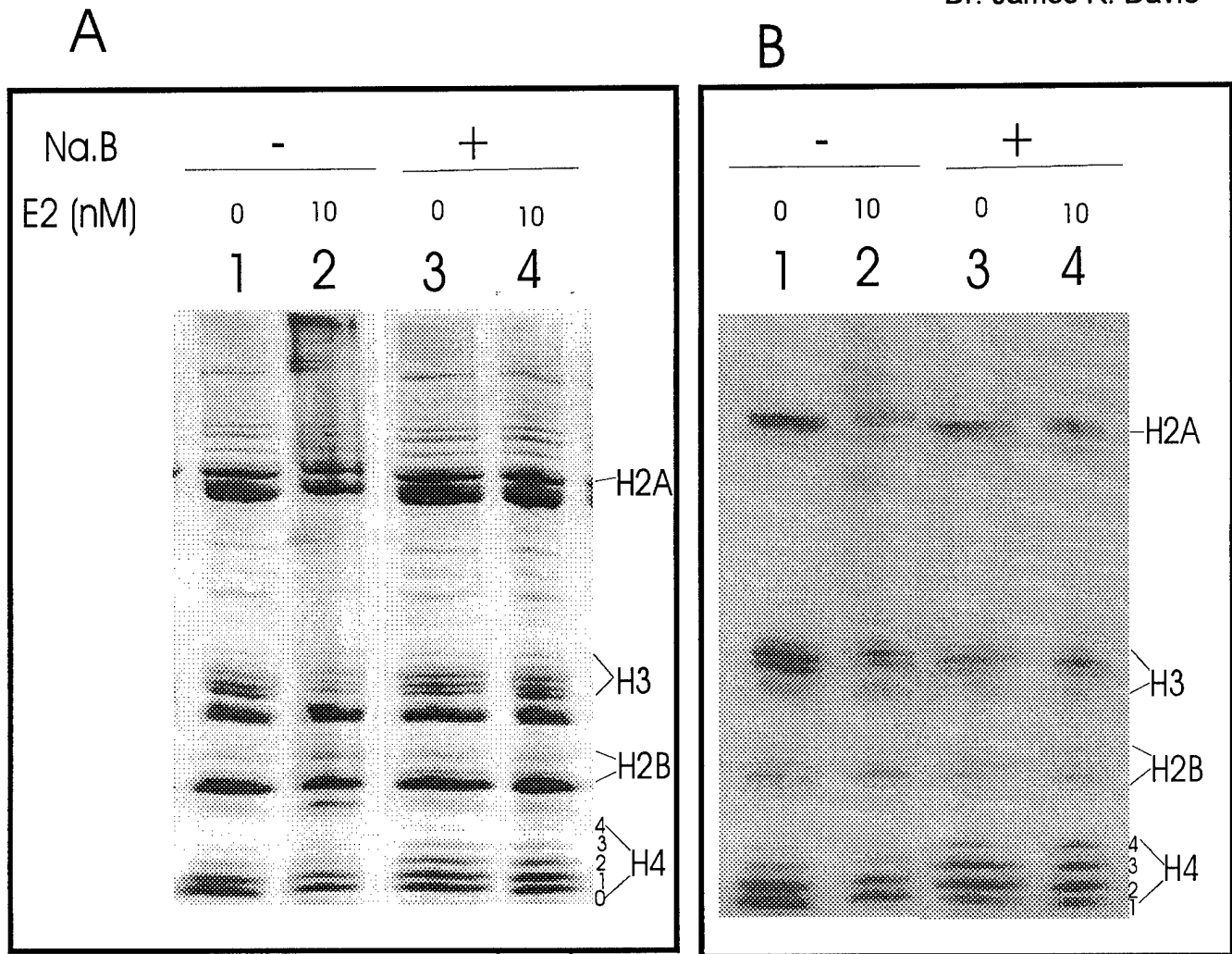


Fig. 2. Estradiol does not affect histone acetylation in human breast cancer MDA MB 231 cells, which are ER negative and hormone independent. The cells were grown in phenol red free DMEM medium containing 7% charcoal stripped FBS for three days, incubated in the presence or absence of 10 nM estradiol (E2) with or without 10 mM sodium butyrate (Na.B) and [3 H]-acetate for 20 minutes. Panel A is Coomassie Blue stained AUT gel (40 μ g of protein in each lane). Panel B is the fluorogram. The acetylated isoforms of histone H4 are indicated. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, tetra-acetyl isoforms of H4, respectively.



Fig. 3. Western blot analysis of the effect of estradiol on the level of ER and HDAC1 in T47D5 cells. The order and time of the addition or removal of the various agents to the cells is shown in Panel A. Two groups of cells were grown in estrogen depleted medium for 3 days. Cycloheximide (10 μ g/ml) was added 30 minutes before labeling to inhibit new protein synthesis. One group of cells was incubated with 10 nM estradiol (+E2) and 10 mM sodium butyrate, and the other group of cells was incubated with ethanol (vehicle) (-E2) and 10 mM sodium butyrate at 37°C for 2 hours. After butyrate removal, the cells were incubated in the same medium without butyrate for 0 to 240 minutes. The cells (0.5×10^6) were lysed in SDS lysis buffer. Ten μ l of cell lysate was loaded on SDS 10% polyacrylamide gel. The resolved proteins were transferred onto nitrocellulose membranes and immunochemically stained with anti-ER α and anti-HDAC1 antibodies (Panel B).

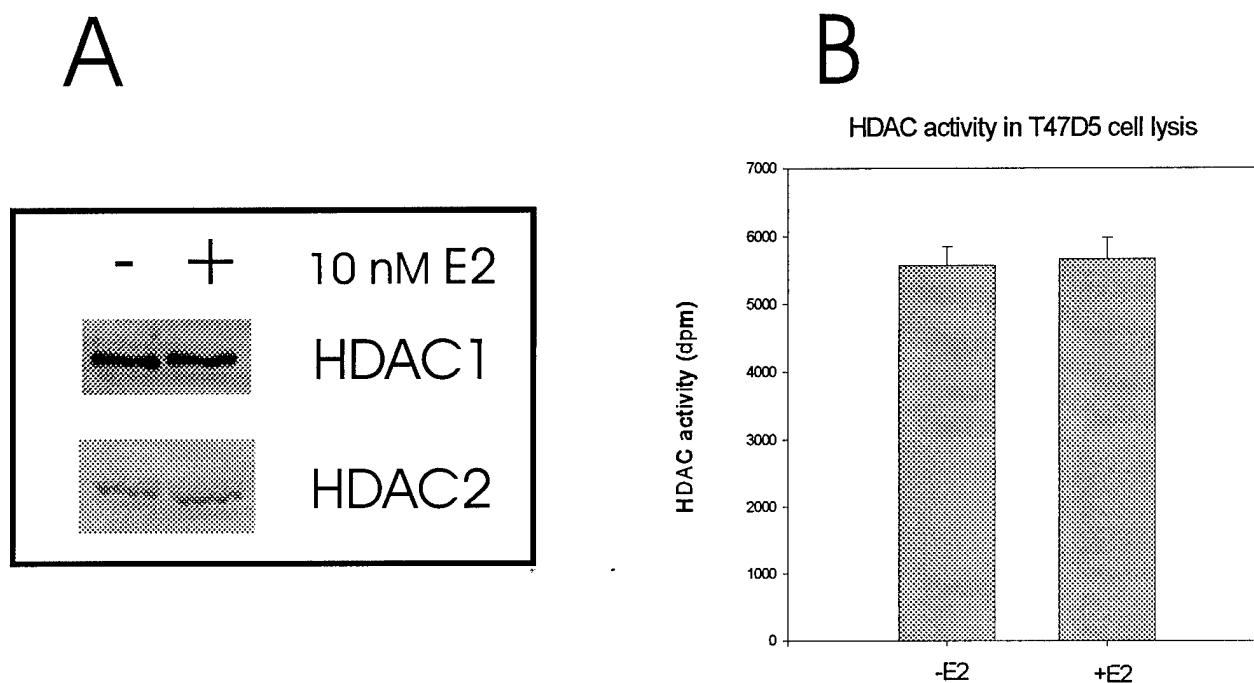


Fig. 4. HDAC level and activity in T47D5 cells treated with and without E2. T47D5 cells were incubated with or without 10 nM E2 for 20 minutes at 37°C. Equal numbers of cells (0.5×10^6) were lysed in TNM buffer with detergent (0.25% NP-40). Equal volumes of cell lysates were resolved by SDS 10% PAGE, and transferred onto nitrocellulose membranes. Anti-HDAC1 and anti-HDAC2 antibodies were used to immunochemically stain the membrane (Panel A). Twenty μ l of cell lysate were used to perform the HDAC activity assays (Panel B).

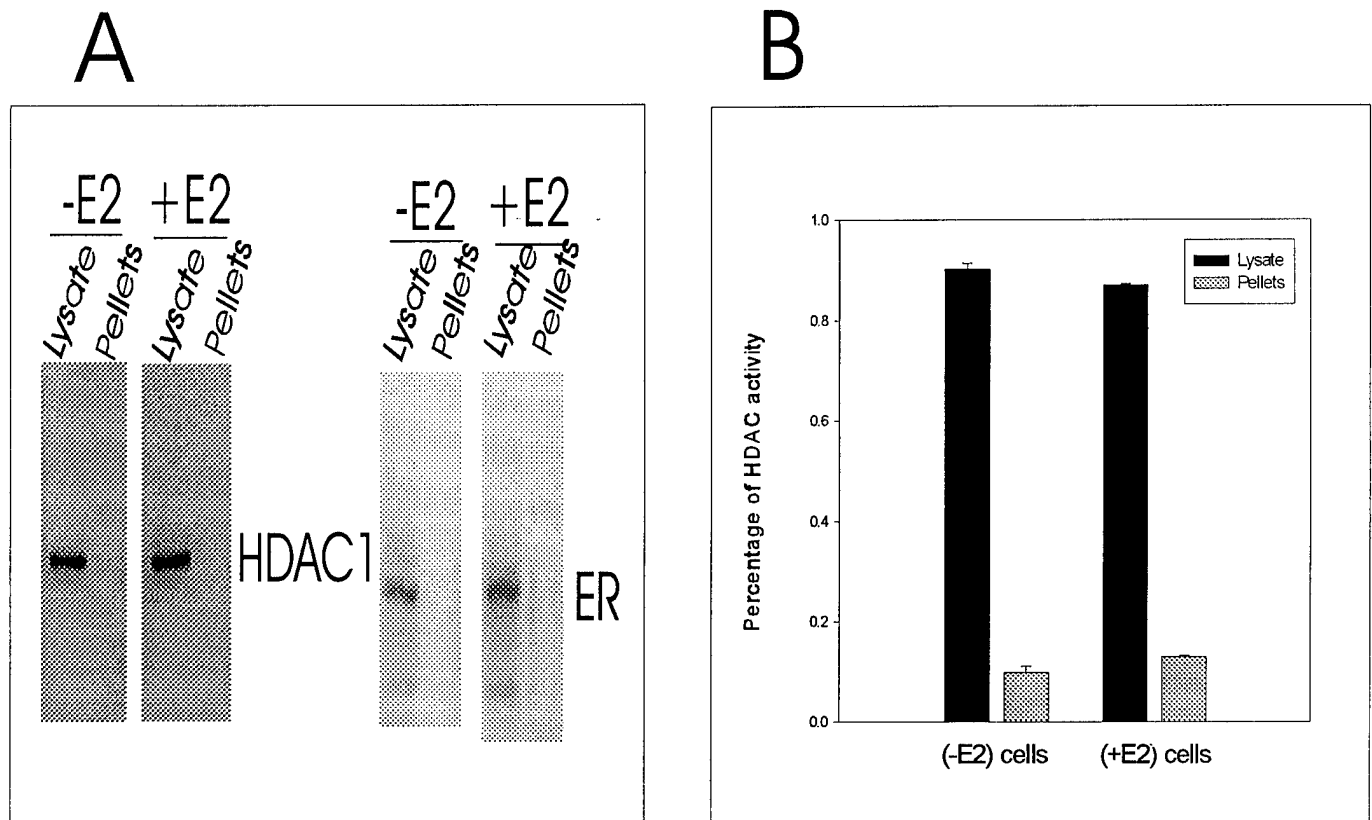


Fig. 5. Efficiency of HDAC1 and ER release from nuclei. T47D5 cells were lysed in IP buffer and sonicated. The supernatant (lysate) and pellets were separated by centrifugation. The pellets were resuspended in equal volume of IP buffer. Equal volume (20 μ l) of cell lysate and pellets were loaded on SDS 10% polyacrylamide gel, and transferred onto nitrocellulose membranes. The antibodies against HDAC1 and ER α were used to immunochemically stain the membranes (Panel A). Equal volumes (50 μ l) of supernatant and pellets were used in the HDAC activity assay. The percentage of total enzyme activity was calculated and plotted (Panel B).

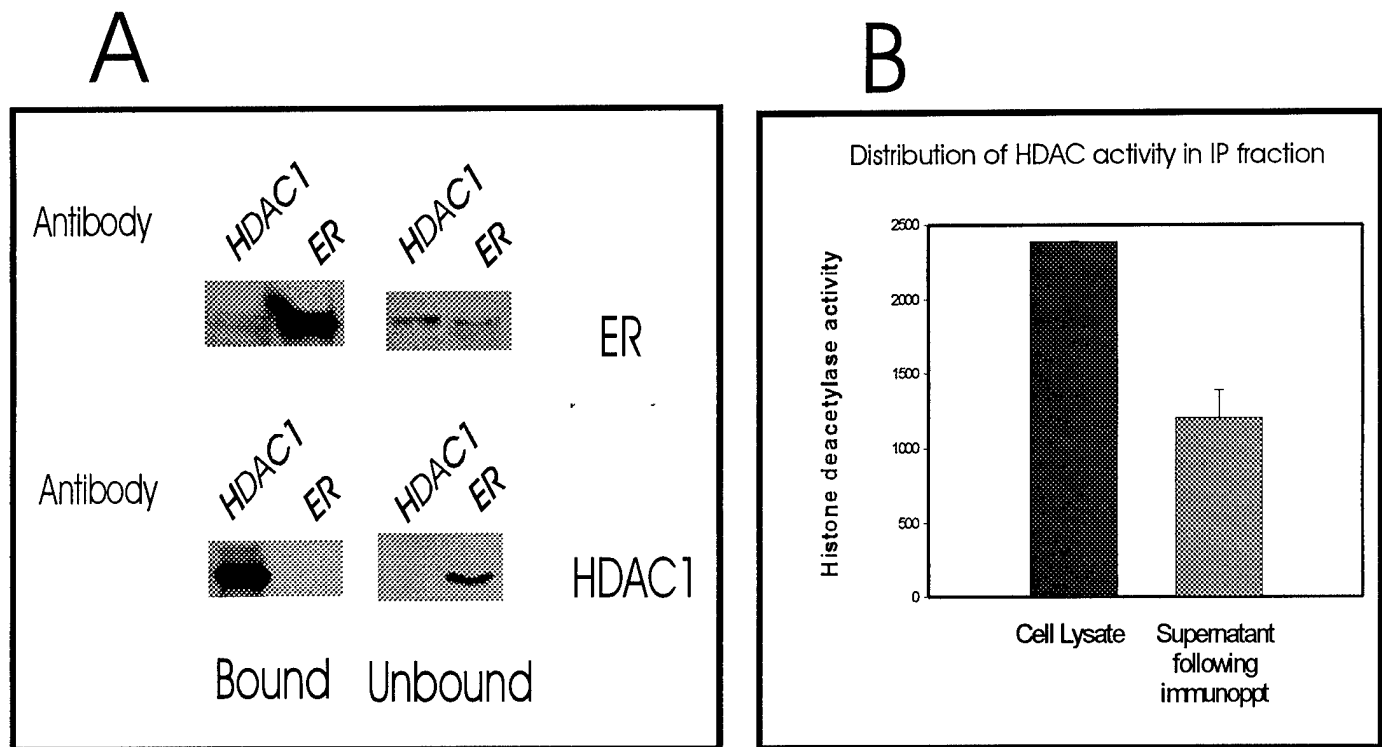


Fig. 6. Efficiency of immunoprecipitation of HDAC1 and ER from T47D5 cell lysates with anti-HDAC1 and anti-ER antibodies. T47D5 cell lysate was incubated with human anti-HDAC1 or anti-ER antibodies. Proteins in the IP (Bound) and unbound fractions were resolved by SDS PAGE, and transferred onto nitrocellulose membranes. Anti-HDAC1 and anti-ER α antibodies immunochemically stained the membranes (Panel A). The HDAC activity in the lysate and immunodepleted fraction was determined. The percentage of total HDAC activity in the fractions was plotted (Panel B).

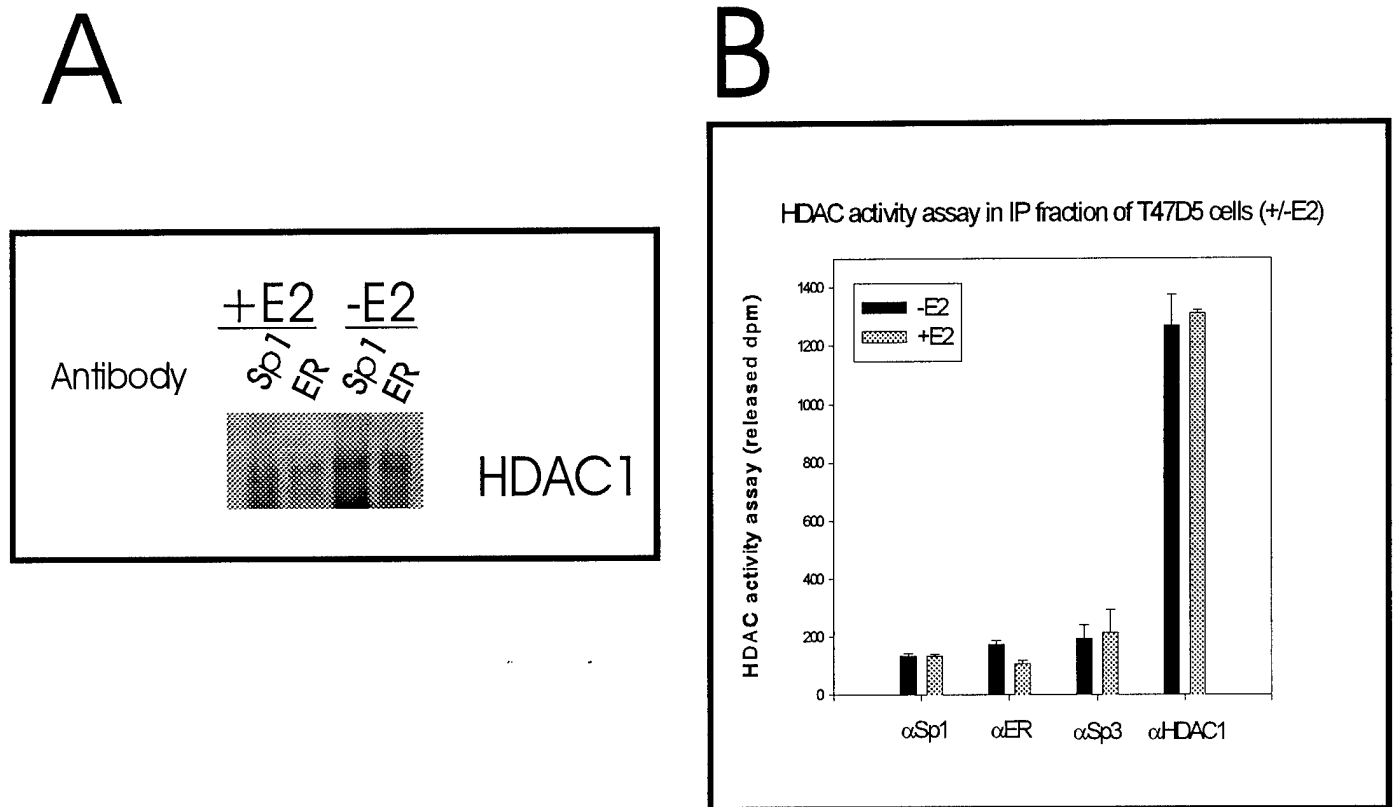


Fig. 7. ER-HDAC1, Sp1-HDAC1 and Sp3-HDAC1 complexes exist in T47D5 cells. The cell lysates from T47D5 cells treated with or without 10nM E2 (at 37°C for 20 minutes) were incubated with anti-HDAC1 or anti-ER α antibodies and protein A-Sepharose. The proteins of the bound and unbound fractions were resolved by SDS PAGE, and transferred onto nitrocellulose membranes. Anti-ER α or anti-HDAC1 antibodies were used to immunochemically stain the membrane (Panel A). The HDAC activities of immunoprecipitated fractions isolated with anti-Sp1, anti-Sp3, anti-ER α and anti-HDAC1 antibodies were assayed. After washing, the Sepharose beads were used in the HDAC activity assay (Panel B).

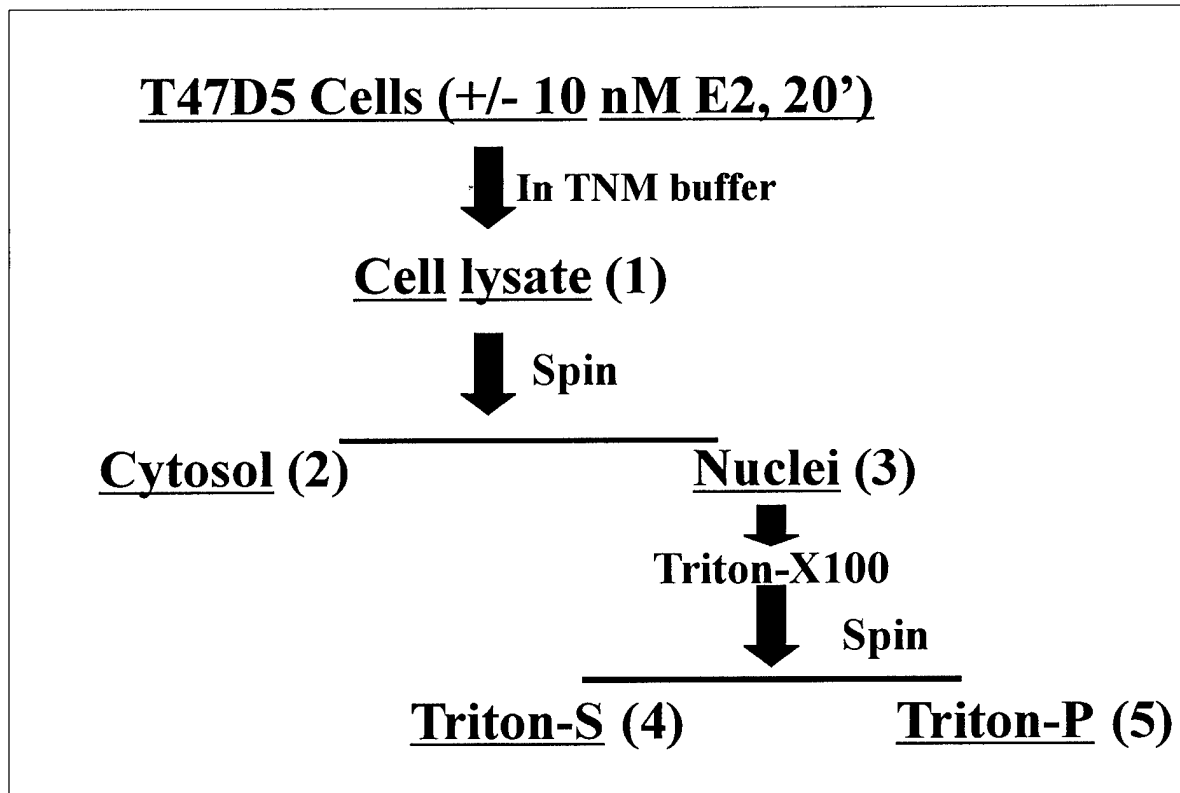


Fig. 8. The flow chart of a novel protocol for cell fractionation. T47D5 cells treated with or without E2 were resuspended in TNM buffer containing no detergent. The cells were lysed by passing through a #22 gauge needle. The cytosol and nuclei were separated by centrifugation. The nuclei were resuspended in TNM buffer with 0.5% Triton X-100, and incubated on ice for 5 minutes. The supernatant and pellets were separated by centrifugation, yielding Triton-S and Triton-P fractions, respectively. The pellets were resuspended in an equal volume of TNM buffer with Triton X-100.

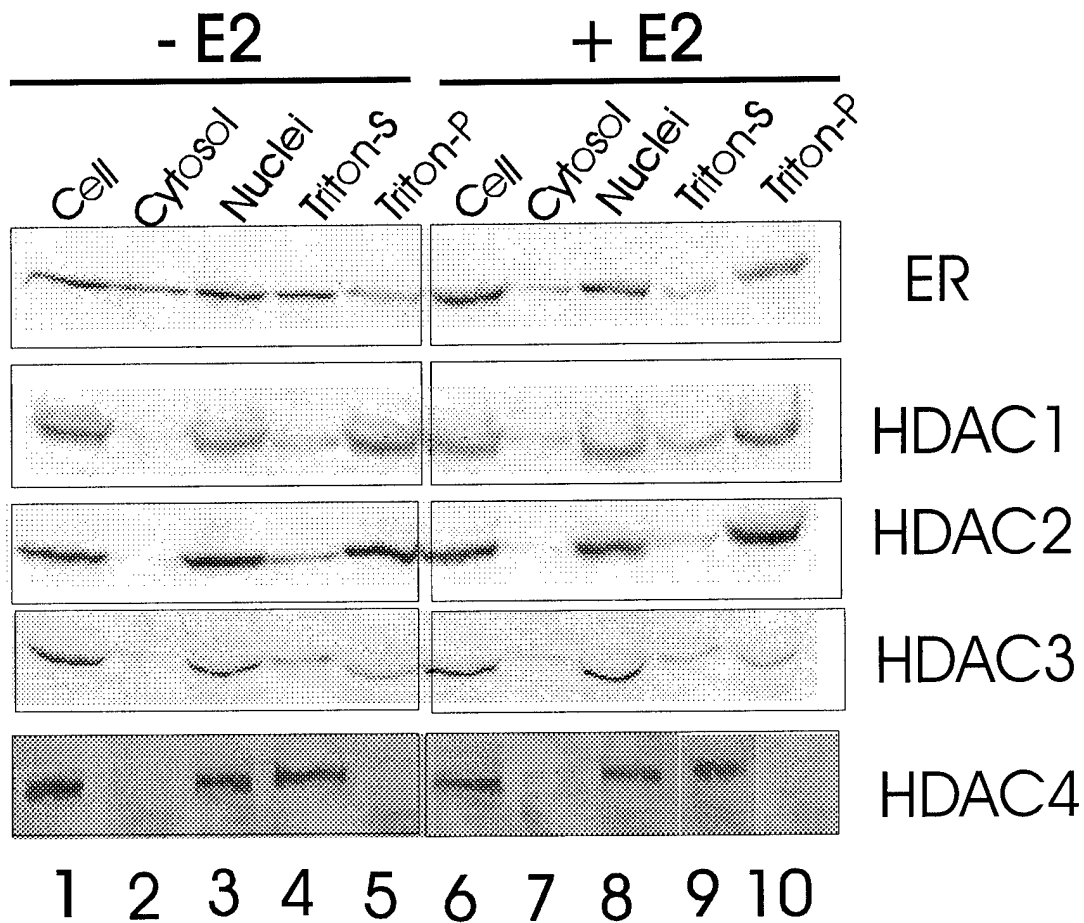


Fig. 9. Distribution of ER and HDACs in cell fractions of T47D5 cells incubated with or without 10 nM E2 (at 37°C for 20 min). Equal volumes (10 μ l) of cell fractions: cell lysate (lane 1 and 6), cytosol (lane 2 and 7), nuclei (lane 3 and 8), Triton-S (lane 4 and 9), and Triton-P (lane 5 and 10), were loaded onto SDS 10% polyacrylamide gels, and transferred onto nitrocellulose membranes. Anti-ER α , anti-HDAC1, anti-HDAC2, anti-HDAC3, and anti-HDAC4 antibodies immunochemically stained the membranes.

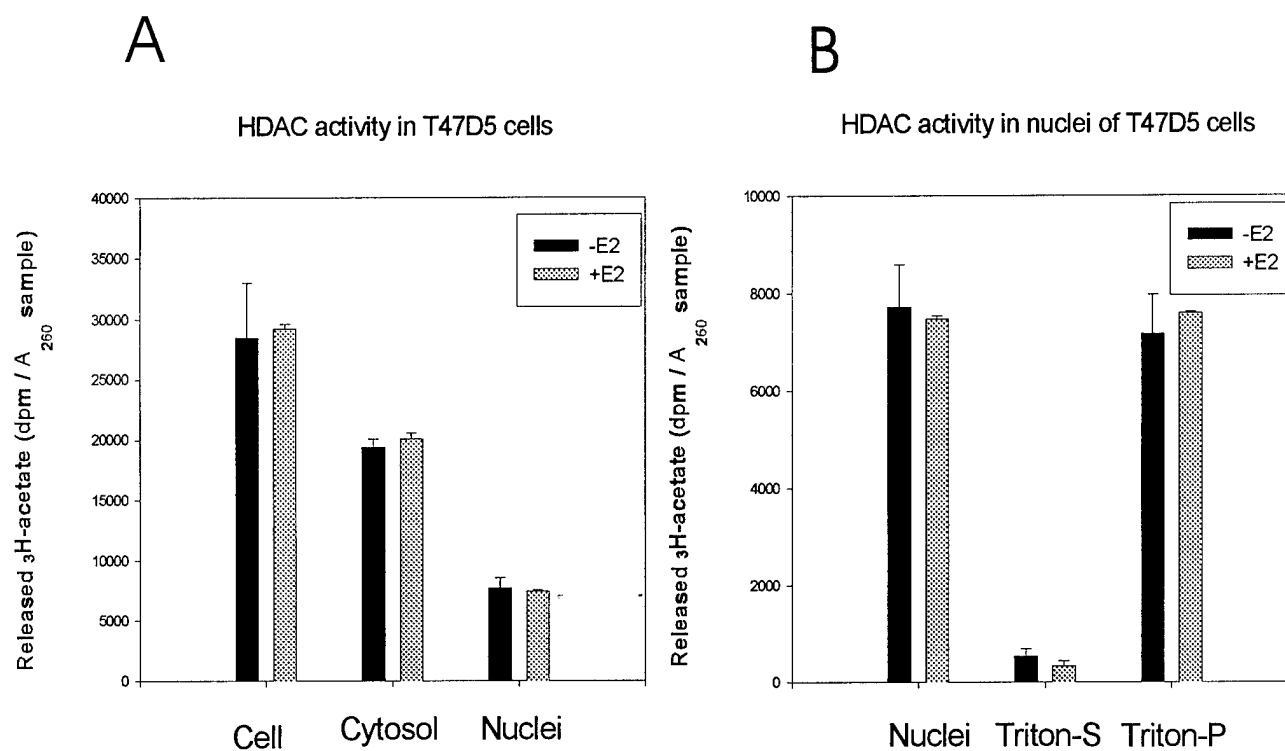


Fig. 10. HDAC activity of cell fractions in T47D5 cells with or without 10 nM E2 (at 37°C for 20 min) treatment. Equal volumes (20 μ l) of cell fractions were used to do the HDAC enzyme activity assays. Panel A is HDAC activity in cell, cytosol and nuclei fractions. Panel B shows the HDAC activity in Triton-S and Triton-P fractions.

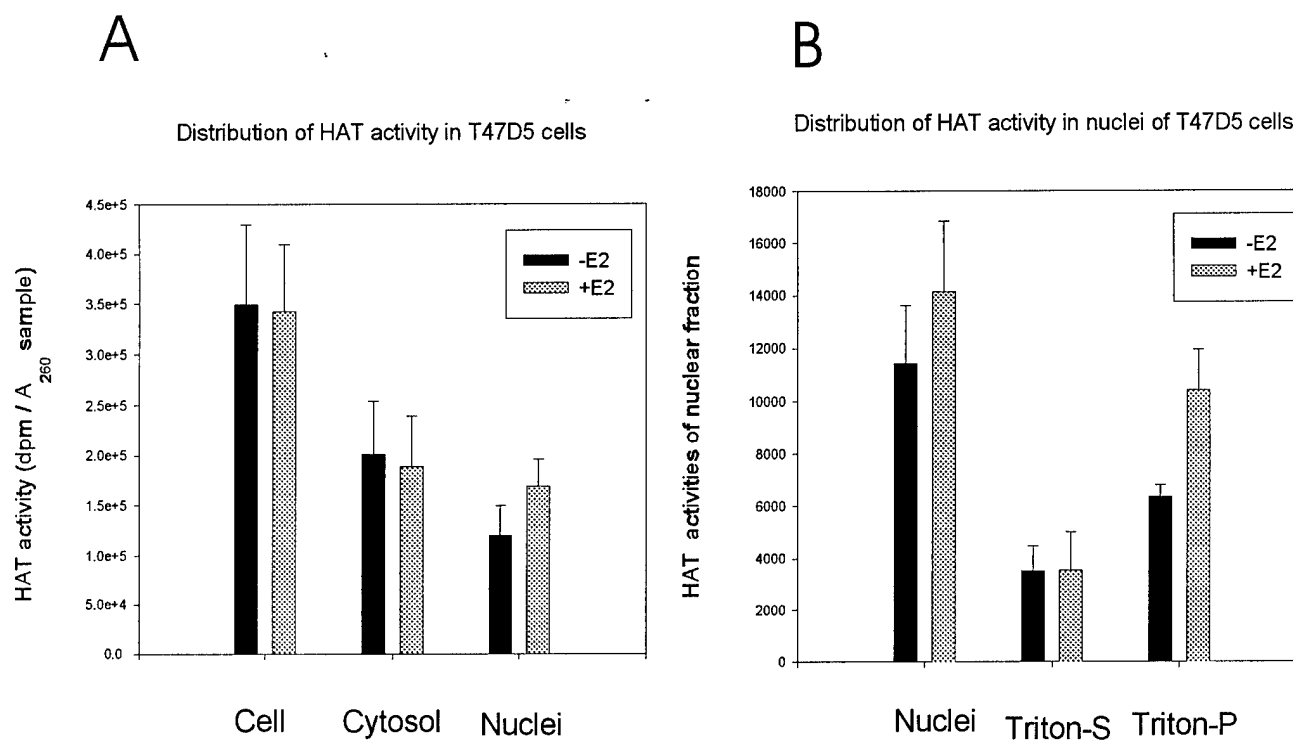


Fig. 11. HAT activity analysis of cell fractions from T47D5 cells treated with or without 10 nM E2 (37°C for 20 minutes). Equal volumes (20 μ l) of each fraction were used to do the enzyme activity assay. Panel A shows the HAT activity in cell, cytosol and nuclei fractions. The distribution of HAT activity in Triton-S and Triton-P fractions is shown in Panel B.

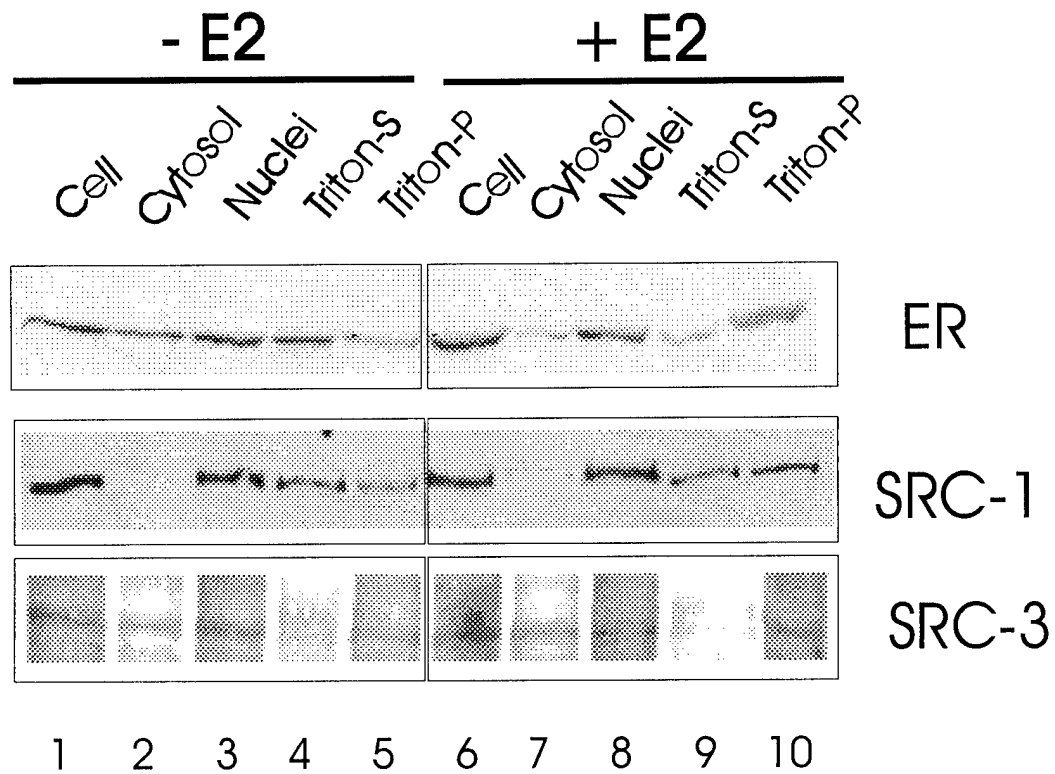


Fig. 12. Partitioning of ER associated cofactors, SRC-1 and SRC-3, in cell fractions from T47D5 cells treated with or without E2 (37°C for 20 minutes). Equal volumes (10 μ l) of cell fractions, cell lysate (lane 1 and 6), cytosol (lane 2 and 7), nuclei (lane 3 and 8), Triton-S (lane 4 and 9), and Triton-P (lane 5 and 10), were loaded onto SDS 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Antibodies against human ER α , SRC-1 and SRC-3 were used to immunochemically stain the membranes.

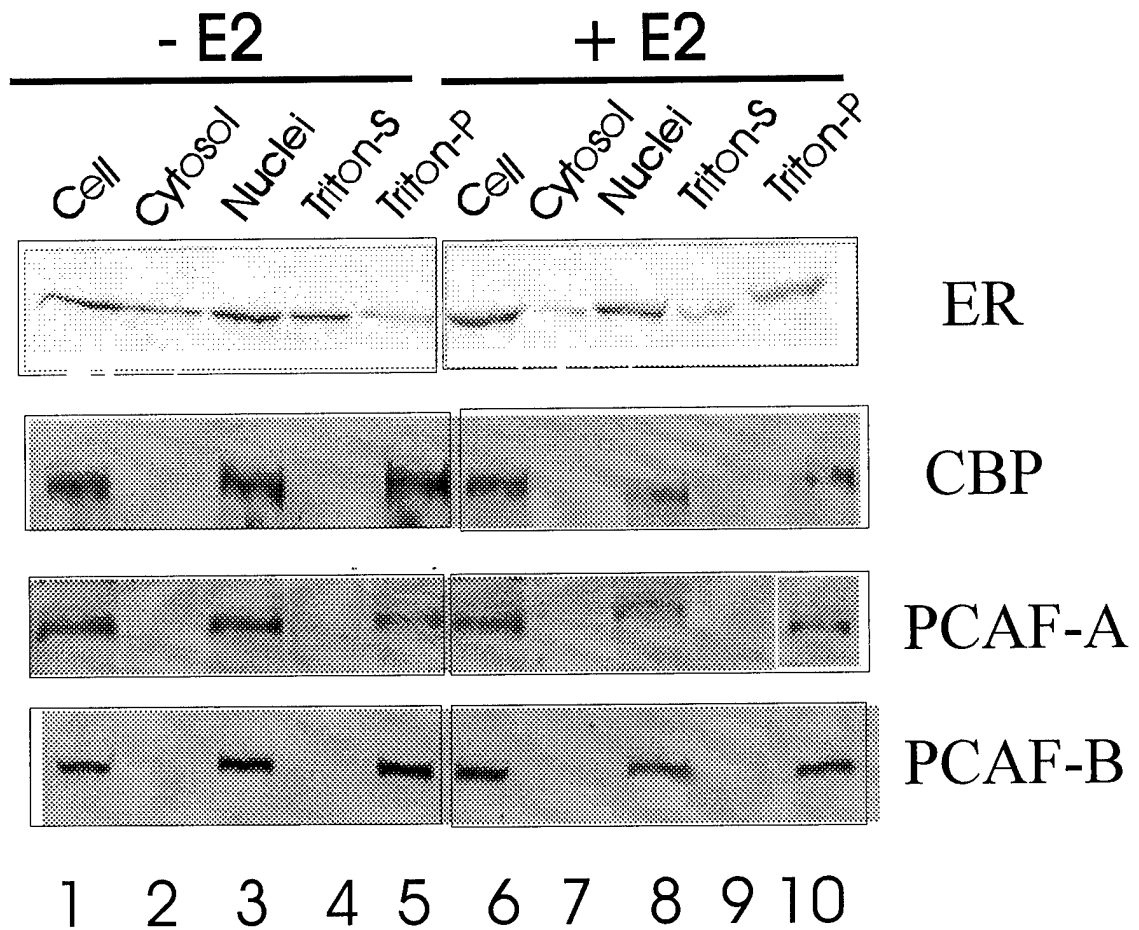


Fig. 13. Distribution of CBP, PCAF-A and PCAF-B in cell fractions from T47D5 cells treated with or without E2 (37°C for 20 minutes). Equal volumes (10 μ l) of cell fractions, cell lysate (lane 1 and 6), cytosol (lane 2 and 7), nuclei (lane 3 and 8), Triton-S (lane 4 and 9), and Triton-P (lane 5 and 10), were loaded onto SDS 10% polyacrylamide gels and transferred into nitrocellulose membranes. Antibodies against human CBP, PCAF-A, PCAF-B were used to immunochemically stain the membranes.

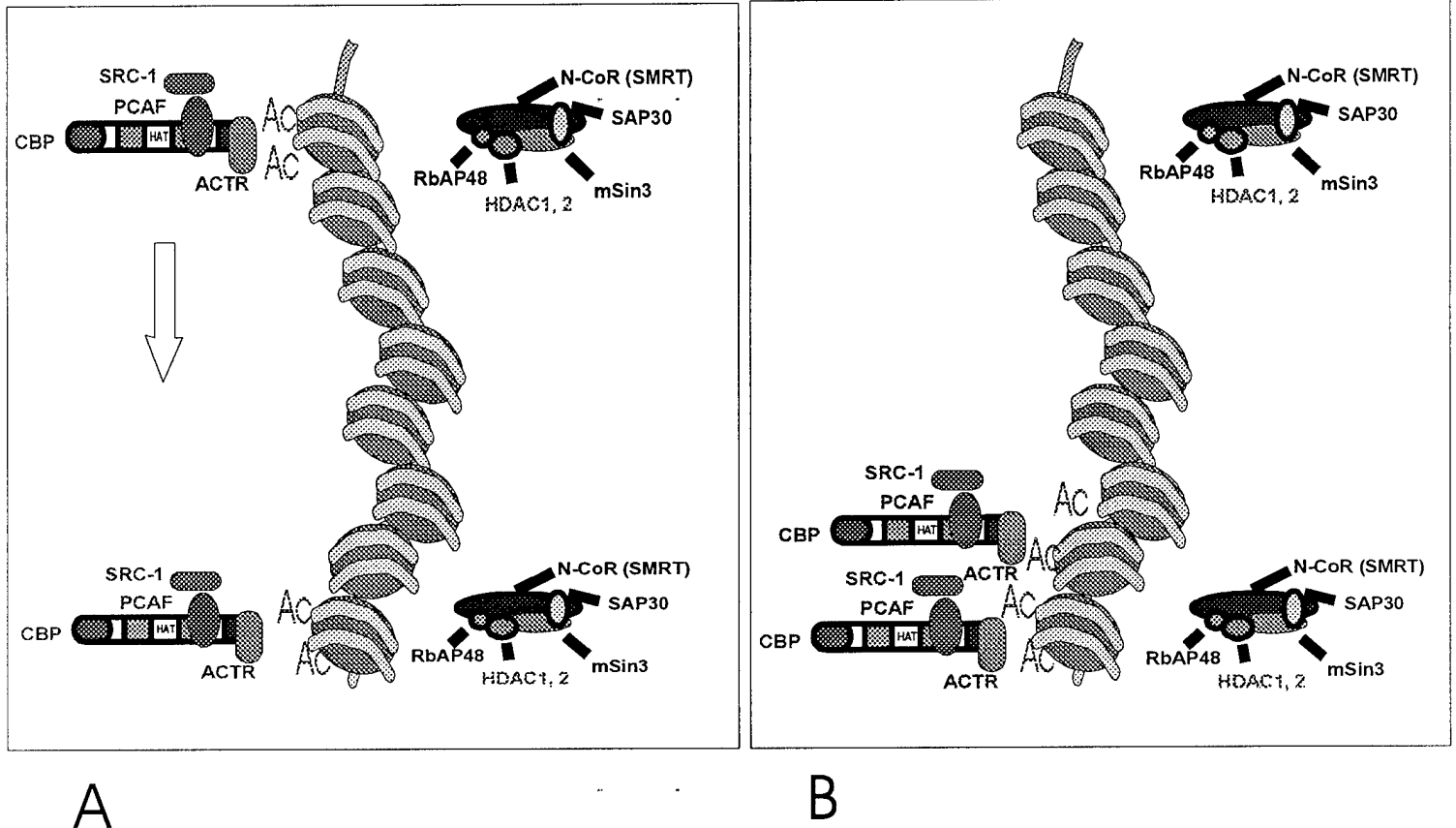


Fig. 14. A model of ER-estradiol mediated recruitment of HATs. When bound to estradiol, ER recruits SRC-1 and SRC-3 (ACTR) from sites also containing HDACs. Without the presence of a HAT, the HDAC is without a substrate and is inactive. At hormone responsive gene promoters where the HATs are recruited, the balance of HATs and HDACs would swing in favor of histone acetylation. Acetylation of histones is increased in regions.

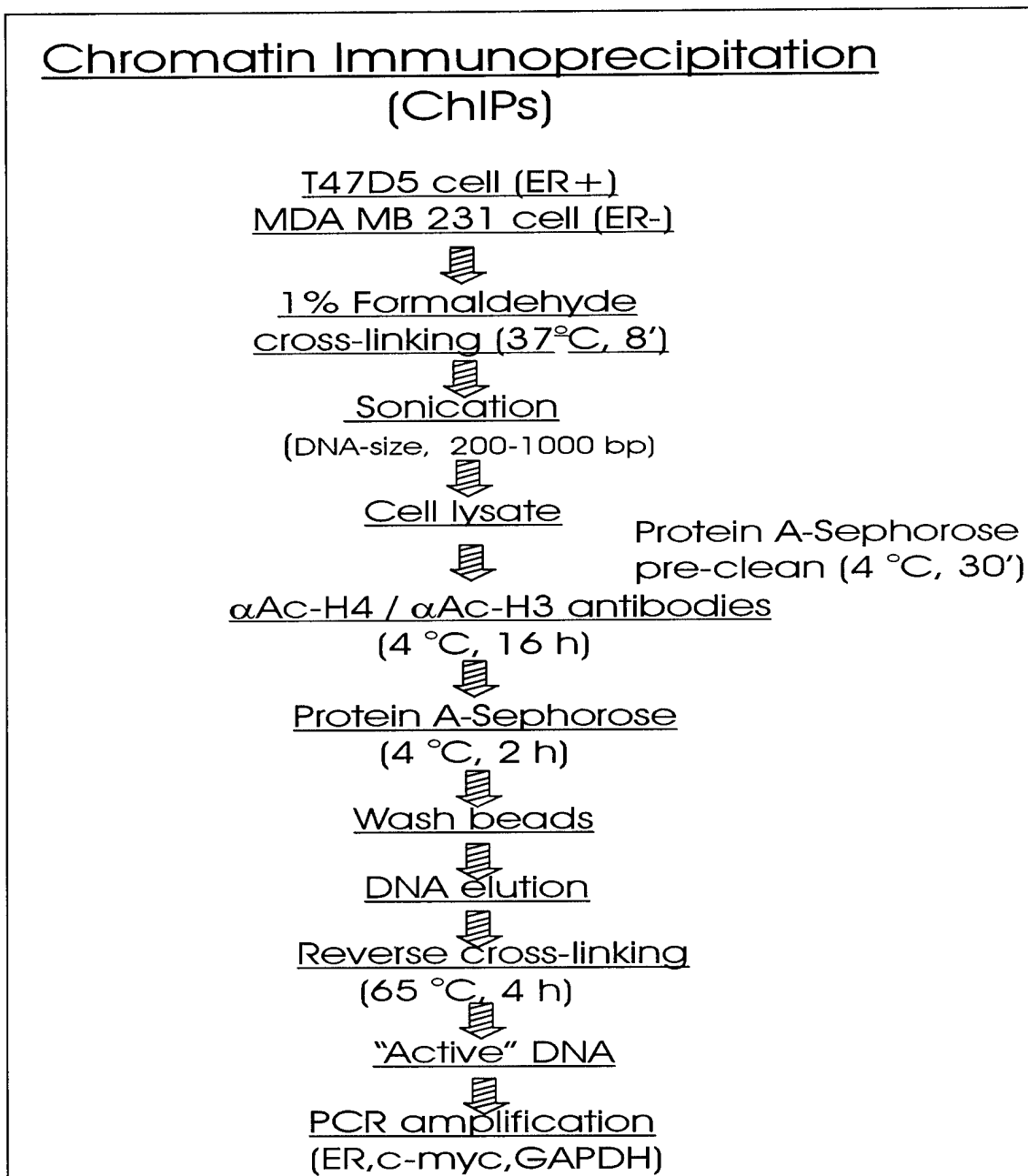


Fig. 15. Flow chart of chromatin immunoprecipitation (ChIPs) protocol. Cells were incubated with formaldehyde to cross-link DNA with proteins. After sonication to cut DNA size to 200-1000 bp, the cell lysate was pre-cleaned with protein A-Sepharose beads. The pre-cleaned cell lysate was incubated with anti-acetylated H4 or anti-acetylated H3 antibodies. Protein A-Sepharose beads were used to precipitate chromatin. The cross-links were reversed and DNA purified. The ChIPs DNA was amplified by PCR with primers monitoring human *c-myc*, ER and GAPDH genes.

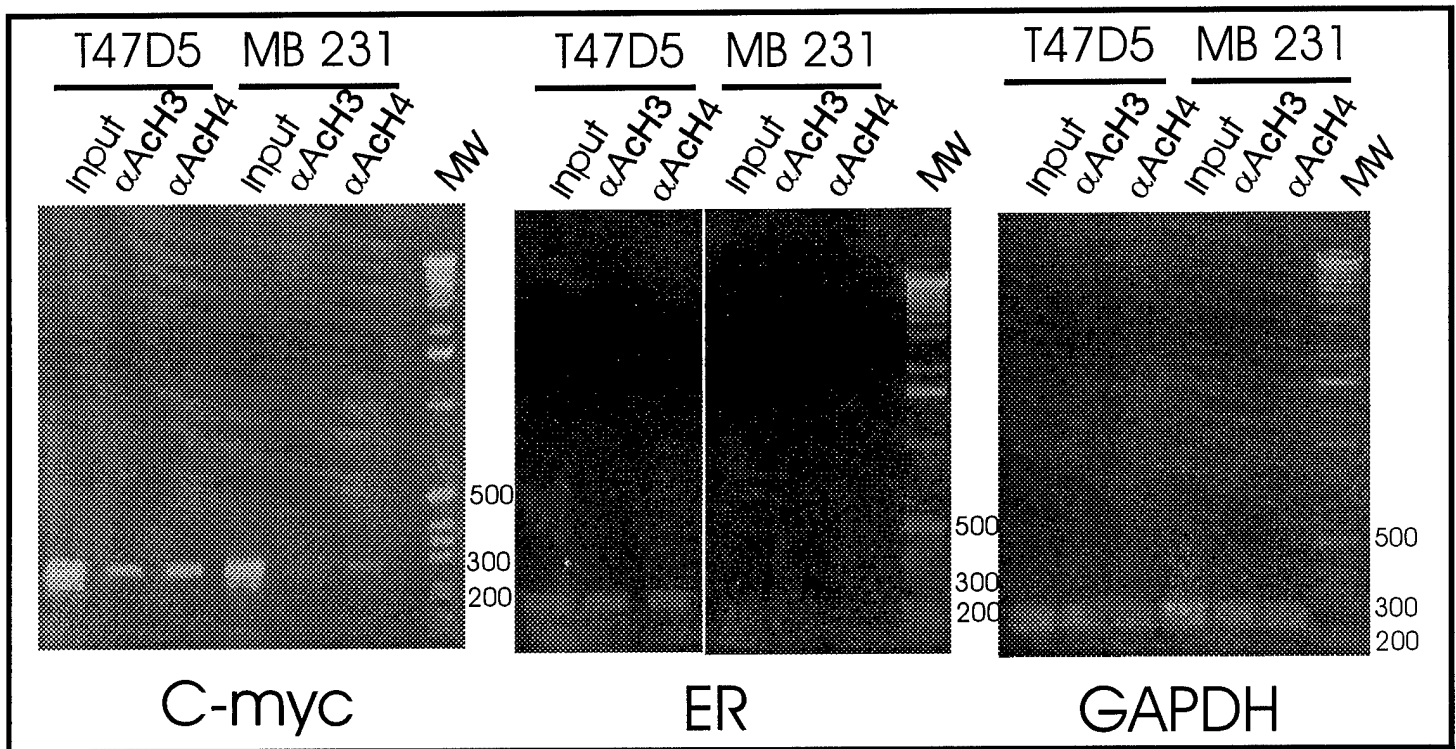


Fig. 16. PCR analysis of DNA fragments isolated by ChIPs from T47D5 cells and MDA MB 231 cells with anti-acetylated H3 and anti-acetylated H4 antibodies. The cell lysates were immunoprecipitated with antibodies against acetylated H3 and acetylated H4 (Upstate). 100 ng of DNA isolated from total cell lysate (input) and isolated by ChIPs with anti-acetylated H3 (α ACh3) and anti-acetylated H4 (α ACh4) antibodies from T47D5 and MDA MB 231 cells were used as template in PCR. The primers, designed from human ER α exon I, human *c-myc* exon I and human GAPDH exon VII, were used in PCR. The PCR products were electrophoresed on a 1% agarose gel. The DNA sizes of PCR products are 279 bp (*c-myc*), 171 bp (ER α) and 284 bp (GAPDH). MW is the DNA molecular markers.

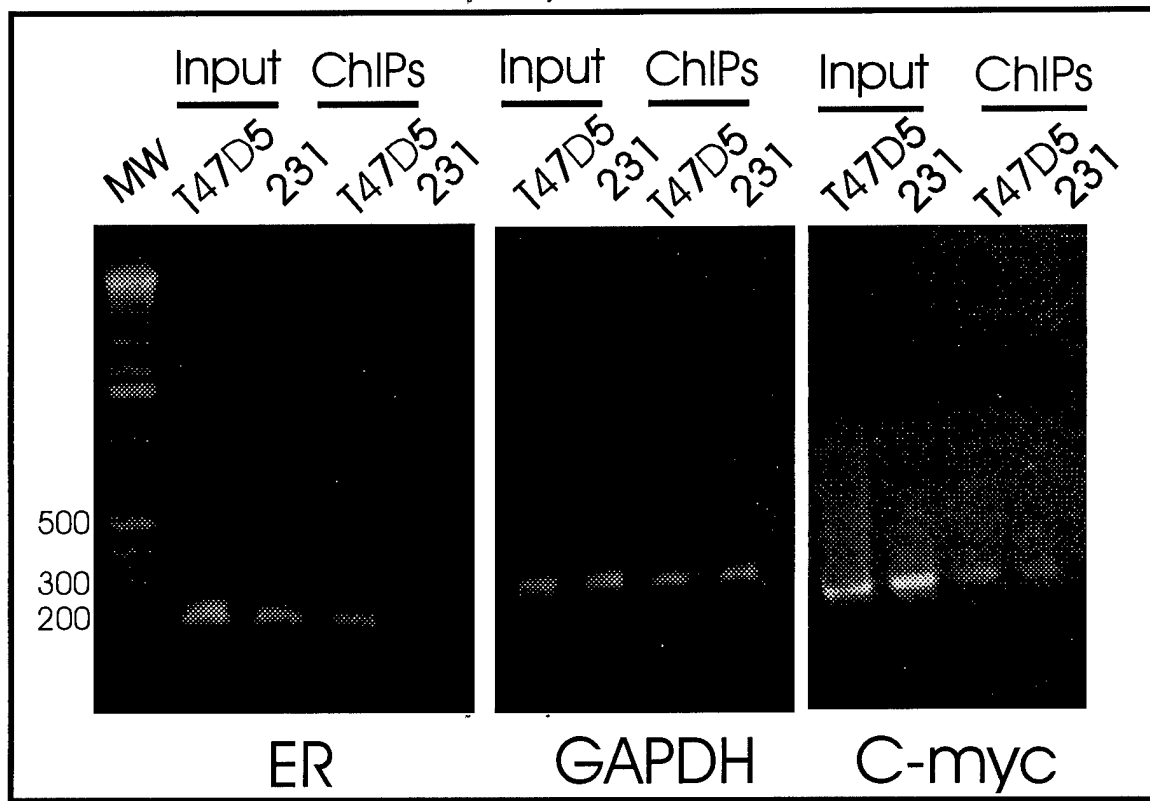


Fig. 17. PCR analysis of DNA fragments isolated by ChIPs from T47D5 cells and MDA MB 231 cells with anti-acetylated H4 antibodies. 100 ng of DNA, isolated from cell lysate (input) and isolated by ChIPs, were used as templates in PCR reactions with primers designed from human *c-myc* exon I, human *ER α* exon I and human *GAPDH* exon VII. The PCR products were electrophoresed on a 1% agarose gel. The DNA sizes of PCR products are 279 bp (*c-myc*), 171 bp (*ER α*) and 284 bp (*GAPDH*). MW is the DNA molecular marker.

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Histone acetylation in human breast cancer cells. The Department of Defense Breast Cancer Research Meeting, Atlanta, page 133, 2000

D. LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT.

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HISTONE ACETYLATION IN HUMAN BREAST CANCER CELLS

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The goal of this research is to establish a protocol to isolate transcriptionally active chromatin from human breast cancer cells. The strategy of this protocol exploits features of transcriptionally active chromatin. Transcribed chromatin is associated with highly acetylated histones that are rapidly acetylated and deacetylated. As the rates of histone acetylation and histone deacetylation in human breast cancer cells have not yet been reported, we did an in depth analyses of the dynamics of histone acetylation in hormone dependent, estrogen receptor positive (T47D5) and hormone independent, estrogen receptor negative (MDA MB 231) breast cancer cell lines. We also studied the effect of estradiol on histone acetylation.

To determine rates of acetylation, T47D5 breast cancer cells were pulse-labelled with [3 H] acetate for 5 or 15 minutes and then chased in the presence of a histone deacetylase inhibitor, sodium butyrate for various times. Histones were electrophoretically resolved on acetic acid-urea-Triton X-100 polyacrylamide gels. Fluorograms of the gels were scanned to determine rates of acetylation. T47D5 and MDA MB 231 breast cancer cells have two rates of histone acetylation. One population of histones, which comprises approximately 10% of the histones, is rapidly hyperacetylated ($t_{1/2}$ = 6 to 10 minutes for monoacetylated H4, H3 and H2B). The bulk of the core histones is slowly acetylated ($t_{1/2}$ = 300 to 400 minutes and 150 to 250 minutes for monoacetylated H4, H3 and H2B in T47D5 and MDA MB 231 cells, respectively). Rapidly hyperacetylated H4 is rapidly deacetylated ($t_{1/2}$ = 6 minutes for tetraacetylated H4).

The level of histone acetylation is increased when T47D5 hormone dependent breast cancer cells are grown in the presence of estradiol. Estradiol does not affect the rates of acetylation in T47D5 cells. However, estradiol decreases the rate of histone deacetylation in T47D5 cells, resulting in the net increase in acetylated histones.

Transcriptionally active DNA associated with highly acetylated histones was isolated from T47D5 and MDA MB 231 cells by a chromatin immunoprecipitation protocol with antibodies to highly acetylated histone isoforms.

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Dear Dr. Davie:

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Control of Chromatin Remodeling

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ABSTRACT

Chromatin structure has a pivotal role in the regulation of gene expression. Transcriptional activation or repression of a gene requires the recruitment of multiple chromatin remodeling complexes. Chromatin remodeling complexes modulate the higher order structure of chromatin, facilitate or hinder the binding of transcription factors, and aid in or prevent the establishment of a transcriptional preinitiation complex. Two types of chromatin remodeling complexes have been extensively studied, ATP-dependent chromatin remodeling complexes and histone modifying enzymes, which include histone acetyltransferases, histone deacetylases and histone kinases. Transcriptional activators and repressors are responsible for recruitment of one or more of these large, multisubunit chromatin remodeling complexes. In this review, the features of the chromatin remodeling complexes and the modes of their recruitment are presented.

KEY WORDS: chromatin remodeling complexes, histone acetyltransferases, histone deacetylase, histone kinases, gene activation, gene repression

I. THE CHROMATIN OBSTACLE AND TRANSCRIPTION

In a mammalian interphase nucleus, chromatin is organised into highly compact chromosome territories. Multiple levels of chromatin fibre condensation are involved in the compaction of chromatin observed in the chromosome territories [for review see (Spencer and Davie, 1999)]. The basic repeating structural unit in chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around a histone octamer core [(H3-H4)₂ tetramer and two H2A-H2B dimers] (Fig. 1) (Luger et al., 1997). Under physiological conditions, the core and linker (H1) histones stabilize higher order chromatin structures. The compaction of chromatin in the chromosome territories is such that DNA sequences are invisible to the transcription machinery.

To gain visibility to the transcription machinery, chromatin must be decondensed and remodeled. Chromatin regions that are prepared or engaged in transcription have an extended, decondensed, and DNAase I-sensitive structure (30 nm fiber). The DNAase I sensitive domain encompasses stretches of DNA upstream and downstream of the transcribed gene. Further, transcriptionally competent and active chromatin sections are positioned at or near the surface of compact chromosome subdomains (Misteli and Spector, 1998; Verschure et al., 1999; Belmont and Bruce, 1994) and these transcribed regions, which are referred to as perichromatin fibrils, are often found near nuclear bodies, such as interchromatin granule clusters (IGCs) and promyelocytic leukemia (PML) nuclear bodies (Hendzel et al., 1998; Misteli and Spector, 1998; Parfenov et al., 2000; Mintz et al., 1999).

Nucleosomes present several obstacles to the transcription process. TFIID, a large multiprotein complex consisting of TATA-binding protein and 8 to 11 TAF_{II}s, directs the formation of the preinitiation complex on both TATA box-containing and TATA-less promoters of RNA polymerase II transcribed genes. Interestingly, the highly acetylated histones of a

promoter-bound nucleosome may aid in the recruitment of TFIID to the promoter. TAF_{II}250, the largest subunit of TFIID, has a double bromodomain module, which has a high affinity for highly acetylated H4 (Jacobson et al., 2000). Thus, once a nucleosome(s) bound at or near the promoter becomes acetylated, the acetylated H4 tail(s) would facilitate the recruitment of TFIID. Nucleosomes positioned on the promoter, however, inhibit the binding of TFIID to promoter DNA; thus the nucleosome has to be remodeled or displaced. Once bound, promoter DNA is wrapped once around TFIID (Coulombe and Burton, 1999). Initiation of transcription cannot proceed until nucleosomes covering the promoter are removed. Benoit Coulombe has presented an interesting model in which TFIID is displaced from the DNA loop by RNA polymerase II holoenzyme, and in this model transcriptional activators could facilitate TFIID displacement, while transcriptional repressors could hinder movement of TFIID (Coulombe, 1999; Coulombe and Burton, 1999). Benoit Coulombe and colleagues have shown that promoter DNA is wrapped almost one full turn around the RNA polymerase II preinitiation complex (Coulombe, 1999; Coulombe and Burton, 1999; Robert et al., 1998). Nucleosomes also impede transcriptional elongation. To counteract nucleosome repression, remodeling is required.

There are two broad groups of chromatin remodeling complexes; complexes that use ATP hydrolysis to increase access of nucleosomal DNA to transcription factors and nuclear enzymes, and histone modifying enzymes, which include histone acetyltransferases, histone deacetylases, and histone kinases.

II. CHROMATIN REMODELING MACHINES

To overcome the nucleosome obstacle for general transcription factors and transcriptional activators to gain access DNA sites in chromatin, cells have a multitude of multiprotein ATP-

dependent chromatin remodeling complexes. Several chromatin remodeling complexes have been identified in yeast [SWI/SNF, RSC (remodeling structure of chromatin), ISW1 (Imitation Switch), ISW2, Ino80], *Drosophila* [dSWI/SNF, ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin accessibility complex), NURF (nucleosome remodeling factor)], and human [hSWI/SNF, NuRD (nucleosome remodeling and deacetylation), RSF (remodeling and spacing factor), ACF] (Peterson and Logie, 2000; Kingston and Narlikar, 1999; Shen et al., 2000). These chromatin remodeling complexes use the energy of ATP hydrolysis to alter nucleosome and chromatin structure, enhancing the binding of transcription factors to nucleosomal DNA-binding sites. Chromatin remodeling factors are involved in both the activation and repression of gene expression.

Yeast SWI/SNF, the first of the chromatin remodeling complexes to be identified, is a large multiprotein complex of 10-15 subunits and 2-megadalton size (four to five times the mass of a nucleosome) (Sudarsanam and Winston, 2000; Kingston and Narlikar, 1999). The complex is an ATPase, utilising ATP to enhance the accessibility of nucleosomal DNA to transcription factors. Although the mechanism by which SWI/SNF remodels a nucleosome is not known, it has been demonstrated that ATP-dependent nucleosome remodeling disrupts histone-DNA contacts without an associated loss of core histones or structural perturbation of histone octamer (Bazett-Jones et al., 1999; Boyer et al., 2000a).

Among the subunits of SWI/SNF are Arp7 and 9 (Peterson et al., 1998; Cairns et al., 1998). These actin-related proteins may regulate transcription and perturb chromatin structure. The domain structurally similar to actin may be important in the function of SWI/SNF (Cairns et al., 1998). Boyer and Peterson have proposed two models for Arp function (Boyer and Peterson, 2000). In one model Arp proteins use ATP binding and hydrolysis to effect molecular changes that regulate the activity of the complex (e.g., dissociation of the complex from nucleosome

arrays). However, mutation studies of *ARP7* and *ARP9* genes do not support a role for ATP hydrolysis in function of chromatin remodeling factors (Cairns et al., 1998). Alternatively Arps could regulate the assembly of chromatin remodeling complex.

SWI/SNF is involved in both gene activation and repression (Sudarsanam et al., 2000). Whole-genome studies show that SWI/SNF controls transcription of a small percentage (about 6%) of yeast genes (Sudarsanam et al., 2000). These studies show that transcriptional activators or repressors recruit SWI/SNF to specific promoters (e.g., Swi5 transcriptional activator recruits SWI/SNF to yeast *HO* promoter) (Sudarsanam and Winston, 2000). Several scenarios have been proposed to explain a role for SWI/SNF in transcriptional activation. In a situation where a transcriptional activator binds poorly to its site in a nucleosome, the activator would recruit SWI/SNF, resulting in remodeling of nucleosome and facilitating the binding of the transcription factor to the DNA site (Sudarsanam and Winston, 2000). Alternatively, for an activator that binds strongly to its DNA site in a nucleosome, SWI/SNF may aid in subsequent recruitment of other transcription factors or members of preinitiation complex or facilitate elongation. Studies with conditional mutants of *snf2* or *snf5* indicate that SWI/SNF is continuously needed during transcription. In a repression mode, SWI/SNF could aid in the binding of a repressor to a DNA site by nucleosome remodeling, or SWI/SNF may revert the nucleosome from the remodeled state to the normal state (Sudarsanam and Winston, 2000). The mode of action of SWI/SNF as an activator or repressor may depend on the net HAT or HDAC activity at the promoter to which it is recruited (Dahiya et al., 2000).

Yeast **RSC** is also a large multiprotein (15 subunits) chromatin remodeling complex (Kingston and Narlikar, 1999). RSC, which is about ten times more abundant than SWI/SNF, is essential for growth. There are two distinct forms of RSC, Rsc1, and Rsc2 (Cairns et al., 1999). The subunits of RSC are similar to those of SWI/SNF and like SWI/SNF RSC contains actin-like

proteins Arp7 and 9 (Cairns et al., 1998). In addition RSC1 and RSC2 contain Rsc1 and Rsc2 subunits, respectively (Cairns et al., 1999). Each protein contains two bromodomains, a bromo-adjacent homology domain, and an AT hook motif, which are essential for RSC function. As the bromodomain of a histone acetyltransferase has been shown to bind acetylated lysines (Winston and Allis, 1999), it has been suggested that bromodomain of RSC1 and Rsc2 recruits or retains RSC at chromatin regions with acetylated histones (Cairns et al., 1999). This scenario is similar to that proposed for TFIID recruitment to the promoter (Jacobson et al., 2000).

Yeast **ISW1** is a four subunit complex. **ISW2** is a two subunit complex, with both subunits being different from those of ISW1. Both ISW1 and ISW2 have nucleosome-stimulated ATPase and nucleosome spacing activities. ISW1, but not ISW2, has nucleosome disruption activity (Tsukiyama et al., 1999). In *Drosophila* ISWI is the subunit with ATPase activity in the chromatin remodeling complexes NURF, CHRAC and ACF. Mutations in the *Drosophila* ISWI result in structural alterations in the male X chromosome, being much shorter and broader than normal (Deuring et al., 2000). This study suggests that ISWI has a role in modulation of higher order chromatin structure.

Ino80 is a 12 subunit, 1 to 1.5 MDa yeast chromatin remodeling complex (Shen et al., 2000). Ino80 was found in a search for yeast genes related to *Drosophila* ISWI. Among the subunits of Ino80 are actin (Act1), three actin-related proteins (Arp4, 5, 8), and Rvb1 and Rvb2, which share homology with the bacterial Holiday junction DNA helicase RuvB. The Ino80 complex has ATP-dependent DNA helicase activity, stimulates transcription, and may be involved in processing of DNA damage.

Like yeast chromatin remodeling complexes, human complexes (**hSWI/SNFa**, **hSWI/SNFB**) contain ATPases BRG1 or hBRM, actin and actin-related protein Baf53 (Cairns et al., 1998). Human SWI/SNF is associated with the nuclear matrix (Reyes et al., 1997; Zhao et

al., 1998). The actin and actin-related proteins may interact with nuclear matrix proteins (Cairns et al., 1998; Rando et al., 2000). Interestingly, human SWI/SNF lacking actin, BAF53 and the SWI2-like ATPase subunit does not associate with the nuclear matrix (Zhao et al., 1998). The glucocorticoid receptor, estrogen receptor, C/EBP- β isoform, and erythroid kruppel-like factor recruit hSWI/SNF to specific chromatin sites (Chiba et al., 1994; Fryer and Archer, 1998; Kowenz-Leutz and Leutz, 1999; Lee et al., 1999) (Fig. 2).

Human **CHRAC** has four subunits: hSWI (hSNF2H), hACF1 and two histone-fold proteins, CHRAC-15 and -17, which bind to DNA but not nucleosomes (Poot et al., 2000). hACF1 is a member of WAL protein family that may target heterochromatin. It is possible, thus, that CHRAC has a role in heterochromatin dynamics. hACF1 also has a bromodomain, which may target CHRAC to acetylated histone tails (Winston and Allis, 1999; Cairns et al., 1999). *Drosophila* CHRAC also contains histone-fold proteins CHRAC-14 and 16, which form a heteromer (Corona et al., 2000). For structural information on the histone-fold, we suggest the following site (<http://genome.nhgri.nih.gov/histones/>). The role of the histone-fold proteins is not known. However, it has been suggested that the proteins stabilise an intermediate of the nucleosome remodeling process, e.g., by serving as acceptor for transiently displaced histones (Corona et al., 2000). Initially it was reported that *Drosophila* CHRAC contained topoisomerase II. A further analysis of the complex argues that topoisomerase is not a subunit of *Drosophila* CHRAC (Corona et al., 2000). Human CHRAC does not have topoisomerase II (Poot et al., 2000).

Human **RSF** is composed of two subunits hSNF2h (human homolog of *Drosophila* ISWI) and 325-kDa protein (LeRoy et al., 1998). Like CHRAC, RSF can convert irregularly spaced chromatin into arrays of periodically spaced nucleosomes. Human **ACF** (also called WCRF) contains hSNF2 and 190-kDa BAZ1A (bromodomain adjacent to zinc finger domain1A,

also named WCRF180, a protein related to the Williams syndrome transcription factor) (Bochar et al., 2000; LeRoy et al., 2000). BAZ1A/WCRF180 has a putative heterochromatin localisation domain, a PHD finger, and a bromodomain. hACF/WCRF complex has ATP-dependent nucleosome remodeling and spacing activities (LeRoy et al., 2000).

Human **NuRD** is an interesting mix of chromatin remodeling activities. This complex contains ATPase chromatin remodeling and histone deacetylase activities. The ATPase activity is carried by the Mi-2 subunit, which is a member of the CHD (chromo-helicase/ATPase-DNA binding) protein family. The chromatin remodeling properties of Mi-2 are distinct from those of ISWI (Brehm et al., 2000). For example, free DNA appears to have a role in the interaction of ISWI with chromatin, while Mi-2 interacts with nucleosomes. Further, in vitro assays show that the direction of nucleosome mobilisation catalysed by ISWI and Mi-2 differs (Brehm et al., 2000; Boyer et al., 2000b).

III. MAPPING OF ACETYLATED HISTONES

Acetylation of the core histones has a key role in the formation and maintenance of the DNAase I sensitive structure (Krajewski and Becker, 1998; Hebbes et al., 1994; Krebs and Peterson, 2000). The core histones have a similar structure with a basic N terminal domain, a central histone fold domain, which mediates histone-histone and histone-DNA interactions, and a C terminal tail (Luger et al., 1997). The core histones are reversibly acetylated at specific lysine residues located in their N terminal tail domains (Fig. 1).

To map the location of acetylated histones along a specific gene or chromatin domain, the chromatin immunoprecipitation (ChIPs) assay has been widely applied. Antibodies to specific acetylated histones (e.g., highly acetylated H3 or H4) and to specific acetylation sites (e.g.,

acetylated K16 of H4) have told us a great deal about the distribution of acetylated histones in chromatin. However, it is important to note that acetylation is a reversible process and the acetylation status of a histone is dictated by the activities of the histone acetyltransferases (HATs) and histone deacetylases (HDACs) accessing the N-terminal tail of that histone.

Generally plant and vertebrate histones that are rapidly highly acetylated are rapidly deacetylated (Waterborg, 1998). In mammalian cells, about 15% (hepatoma tissue culture cells) of the core histones participate in rapid hyperacetylation ($t_{1/2} = 7$ min for monoacetylated histone H4) and rapid deacetylation ($t_{1/2} = 3$ to 7 min). A second population is acetylated ($t_{1/2} = 200$ to 300 min for monoacetylated H4) and deacetylated at a slower rate ($t_{1/2} = 30$ min) (Covault and Chalkley, 1980). In yeast the acetylation status of the core histones is very different from that found in mammalian cells, with the yeast core histones being maintained in a high acetylation state (Davie et al., 1981). Of the possible 34 acetylated lysines, yeast nucleosomes have a steady state level of 13 (Waterborg, 2000). Consistent with the high acetylation state, yeast chromatin is in a DNAase I sensitive state (Lohr and Hereford, 1979). Biophysical and transcriptional analyses show that nucleosomes do not have to be maximally acetylated to prevent higher order chromatin folding. Acetylation of 10-12 of the 26 lysine sites is sufficient to prevent higher order folding and stimulation of transcription by RNA polymerase III (Garcia-Ramirez et al., 1995; Tse et al., 1998). Acetylation of core histone tails increases their alpha-helical content, and this structural change likely affects tail interactions with proteins and/or DNA, thereby destabilizing higher order chromatin organization (Hansen et al., 1998; Wang et al., 2000a).

The first demonstration that acetylated histones were associated with transcriptionally active genes was made by Crane-Robinson and colleagues (Hebbes et al., 1988), using an antibody that recognises the ϵ -acetyllysine residues in histones. Subsequent studies from this group showed that highly acetylated histones were associated with the avian erythrocyte DNAase

I-sensitive β -globin domain, which includes transcriptionally active and competent DNA regions (Hebbes et al., 1994). The end of the DNAase I-sensitive domain is delineated by a marked transition to nucleosomes with hypoacetylated histones. Numerous studies using antibodies to acetylated H3 and H4 have concluded that transcribed genes are associated with acetylated histones, while silenced regions are not [for review see (Davie and Spencer, 2000; Wolffe and Hayes, 1999)]. Applications of the ChIPs technology in fine mapping studies have shown that the promoter regions of transcriptionally active genes are associated with highly acetylated H3 and/or H4, while the coding regions and regions upstream of the promoter are depleted in highly acetylated histones (Parekh and Maniatis, 1999; Krebs et al., 1999). Based on these studies, it is thought that HATs and HDACs undergo a targeted recruitment to the promoter where they alter the acetylation status of a limited number of nucleosomes positioned on the promoter, resulting in a localised perturbation in chromatin structure that enables transcription factors to gain access to their target DNA sequences. However, Groudine and colleagues found that highly acetylated histones, which were detected with an antibody to ϵ -acetyllysine residues, were not restricted to the promoter region of genes responsive to the HS124 enhancer (Madisen et al., 1998). This group also demonstrated that the nuclease-sensitive human β -globin locus was associated with hyperacetylated H3 and H4. Neither the LCR nor transcription was required for the acetylation of the β -globin chromatin domain. However, when the β -globin gene was expressed, there was a pronounced hyperacetylation of H3 in the LCR and β -globin promoter and gene (Schubeler et al., 2000). Similarly, ChIP analyses of the human growth hormone transgene domain with anti-acetylated H3 and H4 antibodies revealed that acetylated H3 and H4 associated with a 32-kb region that included the human growth hormone LCR and expressed human growth hormone gene (Elefant et al., 2000). Lacking in the literature, however, are studies co-mapping chromatin

structural changes as measured by DNAase I sensitivity and distribution of acetylated histones as measured by ChIP assays (Schubeler et al., 2000).

In mammalian cells, highly acetylated histones are not randomly positioned within the nucleus, but are spatially organised. Studies by Michael Hendzel, David Bazett-Jones and colleagues have revealed that highly acetylated histones are enriched near interchromatin granules clusters (IGCs) and promyelocytic leukemia (PML) nuclear bodies [also called nuclear domain 10 (ND10), Kremer (Kr) bodies, PODs (PML oncogenic domains)] (Hendzel et al., 1998; Boisvert et al., 2000). Further, these studies demonstrated that dynamically acetylated histones were enriched on the periphery of IGCs. Several protein-coding genes are found on the periphery of IGCs and PML bodies (Schul et al., 1998; Smith et al., 1999; Jolly et al., 1999). Similar observations have been made with transgenes expressed in zebrafish (Collas et al., 1999).

IV. HAT COMPLEXES AND GENE TARGETING

ChIP assays have indicated that acetylated histones are associated with gene promoters and in some cases with the gene domain. How are HATs recruited to a specific chromatin location? With the purification and cloning of HATs came the realisation that these enzymes are transcriptional coactivators. Specific transcription factors loaded onto promoters recruit the coactivator/HAT resulting in acetylation of the nearby nucleosomal histones [for review see (Wolffe and Guschin, 2000; Cheung et al., 2000a)]. Several HATs have been identified (Sterner and Berger, 2000), and each HAT is a component of a large, multiprotein complex usually exceeding the size of the nucleosome. A description of the HAT complexes follows.

Yeast **SAGA** (Spt-Ada-Gcn5-acetyltransferase, 1.8 MDa) and **Ada** (0.8 MDa) both contain the HAT, Gcn5, and ADA proteins (Ada3 and Ada2). The SAGA complex contains

Ada, Spt proteins [Spt20 (Ada5), 3, 7, and 8], TAF_{II}s (TAF_{II} 90, 68/61, 60, 25/23, 20/17), and Tra-1, a homolog of the TRRAP (human transformation/transcription domain-associated protein)(Grant et al., 1998a; Berger, 1999). TAF_{II}68, which is homologous to human TAF_{II}20 and related in structure and sequence to H2B, is required for the integrity, nucleosomal acetylation and transcriptional enhancing activities of SAGA (Grant et al., 1998a). Yeast TAF_{II}60 and 17 have sequence similarities to H3 and H4 and interact with each other as a heterotetramer through a histone fold. These observations suggest the presence of a histone octamer-like structure within the SAGA complex. The Ada complex is distinct from the SAGA complex and has proteins (e.g., Ahc1) not found in SAGA (Eberharter et al., 1999).

TAF_{II}90, 61/68, 60, 25, and 17 are shared among SAGA and TFIID complexes. In genome wide expression analysis it was determined that these shared TAFs are required for expression of 70% of the yeast genome. Deletion of the TAFs that have the histone-fold structure affect 59 (TAF_{II}17), 18 (TAF_{II}60) and 9% (TAF_{II}61/68) of the genome, suggesting that individual subunits make distinct contributions to transcription (Lee et al., 2000).

Recruitment of SAGA by transcriptional activators with an acidic activation domain results in localized acetylation of nucleosomal substrates *in vivo* and *in vitro* (Grant et al., 1998a; Ikeda et al., 1999; Kuo et al., 1998). Importantly, the transcriptional stimulatory activity of the recruited SAGA complex is dependent upon its HAT activity and is observed only when acetyl CoA is present (Vignali et al., 2000). However, a concern about the interpretation of these studies was raised when it was reported that acetyl CoA stimulated *in vitro* transcription in the absence of histone-reconstituted templates (Galasinski et al., 2000). To address this question, Workman and colleagues reconstituted chromatin templates that had a promoter region with multiple binding sites for Gal4 with Gal4-VP16 and either NuA4, SAGA or NuA3 (Vignali et al., 2000). Both NuA4 and SAGA, but not NuA3, are recruited to the chromatin template by

factors bearing acidic transcriptional activation domains. In the presence of competing condensed chromatin fibers and acetyl CoA, Gal4-VP16 recruited SAGA or NuA4, but not NuA3, resulting in enhanced transcription and acetylation of H3 or H4. This study demonstrated that independent of transcription, activator-dependent acetylation of H3 or acetylation of H4 of a chromatin template occurred and that acetylation of the chromatin template was sufficient to enhance transcription. Further, these authors reported that SAGA acetylated H3 in the first nucleosomes surrounding the promoter.

NuA4 (nucleosomal acetyltransferase of H4) is a large 1.3 MDa eleven subunit complex. Yeast *Esa1* (essential SAS2-related acetyltransferase; the *ESA1* gene is essential for yeast growth) is the catalytic unit of a multiprotein complex NuA4 that acetylates nucleosomal H2A and H4. Tra1, Act3/Arp4, Act1, and Epl1 are subunits of NuA4 (Grant et al., 1998b; Galarneau et al., 2000). Act3/Arp4 is a nuclear actin-related protein that is involved in epigenetic control of transcription and binds to histone tails of H2A, H3, H4 but not H2B. Act1 is cellular actin and is also a component of the nucleosome remodeling complex SWI/SNF. Epl1 is homologous to Enhancer of Polycomb, a modifier of position effect variegation in *Drosophila* (Galarneau et al., 2000).

In chromatin reconstitution experiments, recruitment of NuA4 by factors with acidic transcription activation domains (e.g., Gcn4 and VP16) to a promoter resulted in acetylation of H4 in nucleosomes in a 3 kb region and stimulation of transcription. Thus, NuA4 can reach and acetylate a greater region of chromatin than is observed with SAGA (Vignali et al., 2000). It is conceivable that the Act3/Arp4 subunit of NuA4 enables this HAT complex to acetylate long stretches of chromatin (Galarneau et al., 2000).

The histone acetyltransferases *Esa1* and Tip60 (Tat interacting protein 60) are members of the MYST family of proteins (named after founding members, MOZ, YBF2/SAS3, SAS2, and

Tip60) (Sterner and Berger, 2000). The Nakatani lab has purified a human complex, which contains Tip60, homologous to yeast NuA4 (Galarneau et al., 2000). The Tip60 complex contains RuvB-like proteins, suggesting that this complex has DNA helicase activity (Shen et al., 2000). A HAT with similar properties to yeast NuA4 was isolated from *Tetrahymena*. One difference, however, is that the *Tetrahymena* HAT (80 kDa) appears to exist as a single protein or as a small multiprotein complex (Ohba et al., 1999).

NuA3 (nucleosomal acetyltransferase of histone H3) is a yeast non-Gcn5 containing HAT complex. The Sas3 subunit of NuA3 has HAT activity, which acetylates H3 in nucleosomes. Sas3 is the yeast homolog of human MOZ oncogene. The NuA3 complex contains yTAF_{II}30, which is also found in TFIID, TFIIF and SWI/SNF. Sas3 subunit of NuA3 binds to Spt16, which is a component of yeast CP (Cdc68/Pob3) – FACT (facilitates chromatin transcriptions) complex. Spt16 is identical to Cdc68, which has roles in activation and repression of genes and in regulation of cell cycle. Cdc68/Spt16 is tightly associated with Pob3 (polymerase one binding) in the yeast CP complex. Spt16 and Pob3 co-purify with yeast DNA polymerase α . Mammalian homolog of Spt16 is a component of FACT, a heterodimeric complex that is involved in chromatin remodeling. The authors suggest that NuA3 may have a role in chromatin remodeling during elongation and replication (John et al., 2000).

Yeast **Elongator** is a multisubunit complex that contains Elp3, a 60-kDa protein with HAT activity that can acetylate all four core histones in vitro (Wittschieben et al., 1999). The 3-subunit elongator complex binds tightly to the hyperphosphorylated carboxy-terminal domain of RNA polymerase II, which is responsible for transcriptional elongation. The HAT activity of Elp3 is essential for elongator activity (Wittschieben et al., 2000). There is evidence that H3 may be the preferred substrate of Elp3 in yeast.

Mediator is a large 20-subunit complex in yeast that stimulates basal transcription, transcription by activators, and RNA polymerase II CTD phosphorylation. Included among the subunits are five Srb proteins, seven Med proteins, Cse2, and Nut2. Nut2 has HAT activity (Lorch et al., 2000). This complex preferentially acetylates histone H3. Related Mediator complexes in mammalian cells include DRIP, ARC, and SMCC/TRAP. DRIP, however, does not have HAT activity (Rachez et al., 2000).

CBP/p300, a coactivator, is one of the most potent mammalian HATs, which is capable of acetylating the four core histones in nucleosomes (Sterner and Berger, 2000). High levels of CBP are located in PML nuclear bodies (Boisvert et al., 2000). The HAT activity of CBP is directly involved in stimulating transcription (Martinez-Balbas et al., 1998). CBP is an integrator of multiple signaling pathways, and many factors are in competition to recruit CBP/p300 [for review see (Davie and Chadee, 1998)]. Transcription factors, including hormone receptors, CREB, and fos-jun, loaded onto promoters or enhancers bind directly or indirectly to CBP/p300, recruiting a coactivator with HAT activity. Further, CBP is a component of the RNA polymerase II holoenzyme.

The steroid receptor coactivators **SRC-1** and **SRC-3** (also called p/CIP, ACTR, RAC3, AIB1, and TRAM-1) bind to a variety of nuclear receptors in a ligand-dependent manner (Xu et al., 2000). SRC-1 is a component of a multiprotein complex that contains TIF2 (transcription intermediary factor-2) and SRA, a RNA with coactivator activity (McKenna et al., 1998; Lanz et al., 1999). Both SRC-1 and SRC-3 have HAT activity; however, their HAT activity is weak compared to CBP/p300 and PCAF. These coactivators associate with CBP/p300 and PCAF. Thus, a ligand-activated nuclear receptor could recruit multiple coactivators with HAT activity (e.g., Tip60, SRC-1, CBP and PCAF) (Blanco et al., 1998; Brady et al., 1999).

The acetyltransferase activity of CBP/p300 is not limited to histones (Sterner and Berger,

2000). CBP/p300 acetylates a variety of transcription factors; thus, in addition to being a HAT CBP/p300 is also a transcription factor acetyltransferase (Sternier and Berger, 2000; Cheung et al., 2000a). For example, CBP acetylates p53 and GATA-1 and potentiates the activities of these transcription factors (Berger, 1999). CBP also acetylates other HATs (e.g., ACTR, SRC-1), which disrupts the interaction of the coactivator (ACTR) with the estrogen receptor (Chen et al., 1999).

CBP, SRC-1 and AIB1 are phosphoproteins. CBP is phosphorylated by ERK1, enhancing the HAT activity of CBP in vitro (Ait-Si-Ali et al., 1999). This observation suggests that the activity of CBP may be regulated by the Ras-MAPK pathway (Liu et al., 1999a). Likewise, AIB1 (amplified in breast cancer 1) is phosphorylated by Erk2; this phosphorylation event increases AIB1's affinity for CBP through AIB1's AD1 domain, increasing the transactivation activity of AIB1 (Font and Brown, 2000). SRC-1 is also phosphorylated through the mitogen-activated protein kinase (MAPK) pathway (Rowan et al., 2000). In addition to regulation by phosphorylation, Twist binds to the HAT domain of p300, inhibiting its HAT activities (Hamamori et al., 1999). Twist is a basic helix-turn helix protein that negatively regulates differentiation of multiple cell lineages.

The C terminal domain of human PCAF, a nuclear HAT and transcription factor acetyltransferase (Sternier and Berger, 2000; Cheung et al., 2000a), is similar to yeast Gcn5, while the N terminal domain of PCAF associates with coactivators with HAT activity, e.g., CBP/p300 and SRC-3. Similar to yeast SAGA, human PCAF is in large multiprotein (at least 20 subunits) complex. The PCAF multiprotein complex consists of human counterparts of yeast ADA proteins, Spt proteins, human TAF_{II}s (TAF_{II}30, 31, 20/15, and two TAF-like proteins, PAF65 α and PAF65 β), and PAF400, a 400 kDa protein almost identical to TRRAP (Berger, 1999). Human TAF_{II}31 and 20/15 have the histone fold structure found in histones H3 and H2B,

respectively. PAF65 α has similarity to human TAF_{II}80 and has an H4-like region, while PAF β has similarity to the WD40 repeat-containing TAF_{II}100 (Ogryzko et al., 1998). PAF65 α may associate with hTAF_{II}31 and hTAF_{II}20/15 to form a histone octamer-like structure (Schiltz and Nakatani, 2000). It has been suggested that this histone octamer-like structure displaces a histone octamer. The replaced octamer does not have tails and would be incapable of forming higher order chromatin structures.

PAF400 may have a role in regulating p53 activity following DNA damage. In response to DNA damage by ultraviolet light, PCAF acetylates the C terminus of p53, resulting in enhanced sequence-specific DNA-binding of p53 (Sakaguchi et al., 1998; Liu et al., 1999b). Independent of CBP, the PCAF complex can be directly recruited by several factors, including NF-Y (CCAAT-binding factor), nuclear hormone receptors, and the viral oncoprotein E1A (Blanco et al., 1998; Jin and Scotto, 1998), for review see (Davie and Chadee, 1998).

In mammalian cells, differentially spliced forms of *Gcn5* transcripts generate different Gcn5 isoforms (Smith et al., 1998; Xu et al., 1998). A 98-kDa long form of mammalian Gcn5 has a length similar to PCAF and has 75% identity with this protein. Schiltz and Nakatani suggest that this protein be named **PCAF-B** (Schiltz and Nakatani, 2000). PCAF-B is present in a SAGA-like complex, called STAGA, and in a complex called TFTC (TATA-binding protein-free TAF_{II}-containing complex (Brand et al., 1999). PCAF-B is expressed early in development (mouse) and is expressed uniformly and ubiquitously. Knockouts of PCAF-B are lethal. PCAF is expressed in development later than PCAF-B, and in contrast to PCAF-B knockouts of PCAF are not lethal.

MOF is a component of a multiprotein complex that contains MSL proteins. MOF acetylates K16 of H4 (Akhtar and Becker, 2000). MOF is an X-linked msl protein involved in

acetylating H4 of the male *Drosophila* X chromosome. H4 acetylation is thought to contribute to decondensation of the male X chromosome and increasing its transcriptional activity.

V. MORE HATs YET TO COME

In a search for HATs, responsible for the acetylation of histones within the avian β -globin domain, Hebbes and Allen identified what appear to be novel HATs that acetylate H3 or H4 in transcriptionally active, salt-soluble chromatin (Hebbes and Allen, 2000). The authors speculate that these HATs are responsible for the acetylation of histones along the globin domain. The study did not find an H2B acetylating activity. It is likely that the HAT acetylating H2B is strongly bound to the nuclear matrix (Hendzel et al., 1994). Following removal of 70-90% of the chromatin, the residual avian erythrocyte nuclear material retains the majority of active DNA, HATs and HDACs. This residual nuclear fraction catalyzes dynamic acetylation with the remaining chromatin fragments (Hendzel et al., 1994). Future studies will surely reveal novel nuclear matrix-associated HAT complexes that are involved in the acetylation of histones within DNAase I-sensitive chromatin domains.

VI. HDACs AND CHROMATIN REPRESSION

In contrast to HATs, recruitment of HDACs can lead to repression. Transcription factors associate with corepressor complexes that have HDAC activity. The recruitment of a HDAC multiprotein complex results in deacetylation of nucleosomal histones, leading to the condensation of chromatin (Kouzarides, 1999). However, acetylated HMG proteins and transcription factors may also be targets of the HDAC activity. It is important to note that

chromatin regions engaged in transcription are associated with dynamically acetylated histones [for review see (Davie and Chadee, 1998)]. Thus, both HATs and HDACs are recruited to these regions. When the balance of activity of these two enzymes favors deacetylation, the chromatin region will take on a repressive higher order structure.

VII. HDAC MULTIPROTEIN COMPLEXES

The yeast histone deacetylase **Rpd3** (also known as rpd3p) was first identified as a corepressor of transcription. Six deacetylases have so far been identified in yeast: **Rpd3**, **Hos1**, **Hos2**, **Hos3**, which comprise the class I deacetylases (Rundlett et al., 1996; Grozinger et al., 1999); **Hda1** (also known as hda1p), a class II deacetylase and Silent Information Regulator 2 (**Sir2**) (Rundlett et al., 1996; Grozinger et al., 1999; Imai et al., 2000). These HDACs are found in distinct complexes and display differential sensitivities to histone deacetylase inhibitors. The 600-kDa HDB yeast complex contains Rpd3, which is mildly sensitive to TSA, a noncompetitive histone deacetylase-specific inhibitor. Hda1 is a component of the 300-kDa HDA complex, which is acutely sensitive to TSA (Carmen et al., 1996).

Originally, Sir2 was characterized as an essential component of complexes responsible for silencing heterochromatin at telomeric regions, silent mating type loci, and ribosomal DNA repeats (Tanny et al., 1999; Guarente, 2000). An additional copy of the SIR2 allele attenuates yeast cell life span by 2-fold; mutation of SIR2 significantly reduces lifespan (Kaeberlein et al., 1999). Establishment of heterochromatin at rDNA loci is essential for yeast cell longevity (Guarente, 2000), which is thought to occur through the intrinsic Sir2 HDAC activity (Imai et al., 2000). Sir2 is unique from any characterized HDAC in that (1) it is not affected by TSA, (2) requires NAD for activity, and (3) also contains ADP-ribosyl transferase activity (Imai et al.,

2000). Since the Sir2 counterpart in mouse (mSir2 α) also contains this deacetylase activity, this may be an important enzyme governing transcriptional silencing and aging in higher eukaryotes (Imai et al., 2000; Strauss, 2000).

As with yeast, there are two main groups of mammalian HDACs (Fig. 3). Class I HDACs include HDAC1, HDAC2 or mRPD3, HDAC3, and HDAC8 (Tauton et al., 1996; Yang et al., 1996; Yang et al., 1997; Hu et al., 2000; Van, I et al., 2000). These HDACs are ubiquitously expressed, and display high similarity to yeast rpd3. HDAC1 and HDAC2 are associated in vivo, but do not associate with HDAC3 (Hassig et al., 1998). The second group of histone deacetylases is the class II deacetylases. These HDACs are larger than 80 kDa and exhibit strong similarity to the yeast hda1. Members of this group include HDAC4 or HDAC-A, HDAC5 or mHDA1, HDAC6 or mHDA2, and hHDAC7 (Wang et al., 1999; Miska et al., 1999; Fischle et al., 1999; Grozinger et al., 1999; Verdel and Khochbin, 1999; Kao et al., 2000). HDAC4 and HDAC 5 co-immunoprecipitate with HDAC3; however, no other class I HDAC was found to associate with class II HDACs in vivo in Jurkat cells (Grozinger et al., 1999). Unlike class I, class II deacetylases are expressed in specific tissue cells and/or states of differentiation (Grozinger et al., 1999; Fischle et al., 1999; Verdel and Khochbin, 1999; Kao et al., 2000). Given that yeast Rpd3 (class I homolog) and Hda1 (class II homolog) are in distinct complexes, these mammalian class I and II HDACs may also have distinct biological roles.

Class I and class II histone deacetylases can deacetylate the four core histones. However, in vitro studies show that HDACs have site preferences. For example, yeast HOS3 preferentially deacetylates yeast histones at K5 and K8 of H4, K14 and K23 of H3, K7 of H2A, and K11 of H2B (Carmen et al., 1999). Substrate preference is regulated by components of the multiprotein complexes. For example, free avian HDAC1 preferentially deacetylates H3, but not nucleosomal H3. However, nuclear matrix bound HDAC1 multiprotein complexes preferentially deacetylate

free H2B and deacetylate histones in nucleosomes (Sun et al., 1999).

Mammalian HDAC1 and HDAC2 are in large multiprotein complexes, **Sin3** and **NuRD**. The Sin3 complex, which has been estimated size of 1-2 MDa, contains mSin3, SAP18, SAP30, retinoblastoma associated proteins (RbAp) 46 and 48, and c-Ski (Nomura et al., 1999; Ayer, 1999; Knoepfler and Eisenman, 1999). RbAp46 and RbAp48 are also subunits of CAF1 (chromatin assembly factor 1). RbAp46 and RbAp48 can directly bind histones H2A and H4 in vitro, and may aid in tethering the HDAC complex to the histones (Verreault et al., 1998). Currently, there is disagreement whether the Sin3 complex contains the corepressors N-CoR and SMRT (Hassig et al., 1997; Zhang et al., 1998; Li et al., 2000; Cohen et al., 2000; Guenther et al., 2000).

The Sin3 complex is directed to its target chromatin location by sequence specific DNA binding proteins that interact directly with mSin3 and other components of Sin3 complex. When the yeast Sin3-Rpd3 HDAC complex is recruited to a repressed promoter, histone deacetylation occurs over a 1-2 nucleosome range (Kadosh and Struhl, 1998). Some examples of DNA binding proteins that recruit the Sin3 complex include the Mad-family proteins, N-CoR/SMRT-binding unliganded hormone receptors, MeCP2, p53, REST, and the Ikaros-family proteins (Ayer, 1999; Knoepfler and Eisenman, 1999) (Fig. 3). SAP30, which binds to mSin3 and N-CoR, is required for N-CoR/mSin3 mediated repression of hydroxytamoxifen-bound estrogen receptor and homeodomain protein Rpx, but not unliganded retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (Laherty et al., 1998; Zhang et al., 1998) (Fig. 2). Interestingly, microinjection of anti-N-CoR or anti-SMRT IgG into cells can convert hydroxtamoxifen-bound estrogen receptor and RU486-bound progesterone receptor from repressors to activators (Lavinsky et al., 1998).

Another complex called NuRD (nucleosome remodelling histone deacetylase complex) is about 2 MDa in size and consists of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, RbAp46/48 and MBD3 (methyl-CpG-binding domain-containing protein). NuRD has both ATP-dependent chromatin remodeling and HDAC activities (Zhang et al., 1999). ATP stimulation of deacetylation of chromatin templates by NuRD vary from no stimulation to about 3-fold.

CHD3 and CHD4 (also known as Mi-2 α and Mi-2 β respectively) are members of the CHD family of proteins. The CHD protein family contains a chromodomain and an ATPase/DNA helicase domain present in the SWI/SNF family. Originally, CHD3 and CHD4 were identified as self-antigens found in dermatomyositis patients. MTA2 is 65% identical to MTA1, which is found at elevated levels in several metastatic cell lines and tissues. MTA2 expression was also increased in cervical cancer tissue. MTA2 modulates the HDAC activity found in the core unit (HDAC1/2, RbAp46/48), but its exact function within the NuRD remains elusive (Ayer, 1999; Knoepfler and Eisenman, 1999).

HDAC3 is in a 1.5-2 MDa multiprotein complex that has N-CoR and SMRT (Guenther et al., 2000; Wen et al., 2000; Li et al., 2000) (Fig. 3). N-CoR also binds to HDACs 4, 5 and 7. However, in HeLa cells the majority of the N-CoR and SMRT complexes associate with HDAC3. It has been suggested that HDAC3 is the principle HDAC for the HDAC-dependent repression mediated through N-CoR and SMRT (Li et al., 2000; Guenther et al., 2000). Interestingly, the SMRT-HDAC3 complex contains transducin β -like protein 1 (TBL1). The gene encoding TBL1 gene is mutated in human sensorineural deafness (Guenther et al., 2000). TBL1 is structurally similar to global repressor Ssn6/Tup1 and *Drosophila* Groucho corepressor and like these proteins TBL1 binds to histone H3 (Grunstein, 1998; Edmondson et al., 1996; Palaparti et al., 1997; Fisher and Caudy, 1998). In addition to being recruited by unliganded

hormone receptors, N-CoR and SMRT are also corepressors for the leukemogenic fusion protein PLZF, Notch-binding protein CBF-1, homeodomain proteins (Rpx2, Pit-1 and Rbx), MAD, DAX1, COUP-TF, and Rev-Erb (Glass and Rosenfeld, 2000) (Fig. 3). Deletion of HDAC3 in the DT40 chicken B cell line is lethal (Takami and Nakayama, 2000).

HDACs are recruited to specific genomic sites by transcription factors (repressors). HDAC1, 2 and 3 bind to YY1, while Rb and E2F form a complex with HDAC1 [for review see (Davie and Chadee, 1998; Kouzarides, 1999)]. The IXCXE motif located in the C terminal region of HDAC1 and HDAC2 is important for the association of these enzymes with the Rb “pocket” domain of hypophosphorylated Rb, p107 and p130 (Magnaghi-Jaulin et al., 1998; Ferreira et al., 1998). Mutation of the Rb LXCXE binding site prevents the binding of HDAC1 and 2, but not 3, which lacks the LXCXE-like sequence. Mutation of this site compromised Rb’s ability to repress cyclin E and A promoters and to sustain suppression of growth; however, the binding and repression of E2F activity was not affected by this mutation (Dahiya et al., 2000).

HDAC4 and HDAC5 are found in both the nucleus and cytoplasm (Wang et al., 2000b). 14-3-3 proteins negatively regulate the nuclear localization of HDAC4 and HDAC5. It is speculated that phosphorylation of 14-3-3 proteins regulates its interaction with 14-3-3 proteins, allowing HDAC4 and HDAC5 to locate to the nucleus (Grozinger and Schreiber, 2000; Wang et al., 2000b). Once released into the nucleus, HDAC4 may be recruited by myocyte enhancer factor 2 (MEF2), resulting in the repression of MEF2 transcriptional activation. MEF2 also binds to the co-repressor MITR (MEF-2 interacting transcription repressor), which shares sequence similarity with class 2 HDAC family members. MITR binds to HDAC1; thus, MEF2 is able to recruit both class 1 and class 2 HDACs (Sparrow et al., 1999). Similar to the situation with E2F1, MEF2 associates also with the coactivator/HAT CBP/p300. Thus, the association of MEF2 with co-repressors or coactivators governs the transcriptional response of MEF2 (Miska et

al., 1999).

VIII. DNA METHYLATION AND HISTONE DEACETYLATION

DNA methylation is associated with gene silencing, chromosome X inactivation and imprinting. Histone deacetylases have a role in transcriptional silencing by DNA methylation. Methyl-CpG-binding protein 2 (MeCP2) binds to Sin3, recruiting the Sin3 complex to methylated DNA (Nan et al., 1998; Jones et al., 1998). Mutations in the X-linked gene encoding MeCP2 are the defects of some cases of Rett syndrome, a progressive neurodevelopmental disorder that is a major cause of mental retardation in females (Amir et al., 1999). The lack of stable repression of methylated chromatin may be involved in Rett syndrome. Methyl-CpG-binding domain-containing protein (MBD2) recruits Sin3 and NuRD complexes, providing another avenue for coupling DNA methylation and histone deacetylation in gene silencing (Zhang et al., 1999; Ng et al., 1999; Boeke et al., 2000). MBD2 is also a demethylase (Bhattacharya et al., 1999). Thus, this protein may be involved in de-repression. However, other groups have not been able to demonstrate that MBD2 has demethylase activity (Li, 1999).

HDAC1 is targeted to newly methylated sites through its interaction with Rb. In addition to binding E2F1, Rb also binds to the mammalian DNA methyltransferase, DNMT1 (Robertson et al., 2000). DNMT1, which is the principle enzyme in the maintenance of mammalian DNA methylation, is localized in replication sites, which are associated with the nuclear matrix (Ma et al., 1998). DNMT1 also binds to HDAC2 and a transcriptional repressor, DNMT1 associated protein (DMAP1) (Rountree et al., 2000). DNMT1 recruits HDAC2 only during late S phase of the cell cycle. Transcriptionally repressed chromatin regions such as the inactive X chromosome, heterochromatic regions and transcriptionally silent alleles of selected imprinted

genes replicate late in S phase. These condensed chromatin regions are methylated and associated with hypoacetylated histones. The coupling of HDAC1 and HDAC2 with DNMT1 provides a mechanism for temporally coupling histone deacetylation with methylation during replication. Without this histone deacetylation event the compaction of newly replicated chromatin is compromised (Annunziato and Seale, 1983).

IX. MULTITASKING OF COMPLEXES

Transcription factors may have multiple binding sites for different types of chromatin remodeling factors. An example is Rb, which has separate interaction domains for HDACs and BRG1 (SWI/SNF). Rb needs to bind both HDAC and BRG1 to effectively suppress growth (Dahiya et al., 2000; Strobeck et al., 2000). Rb also binds DNMT1 (see above). The hypophosphorylated form of Rb is associated with the nuclear matrix (Mancini et al., 1994; Durfee et al., 1994). It is possible that hypophosphorylated Rb recruits multiple chromatin remodeling activities, e.g., HDAC1, HDAC2, E2F, SWI/SNF, DNMT1, to specific nuclear sites.

Phosphorylation of Rb by CDK4 and CDK6 results in the dissociation of Rb from HDAC1, E2F and the nuclear matrix (Harbour et al., 1999). The promoter associated E2F may now associate with CBP/p300, recruiting a HAT activity that stimulates transcription of the cyclin E gene, allowing progression into S phase. However, cyclinD-cdk4 phosphorylation of Rb does not affect the binding of BRG1 to Rb. The Rb-hSWI/SNF complex inhibits the expression of cyclin A and cdc2 kinase, preventing movement of cells into M phase. Subsequent phosphorylation of Rb by cyclin E-CDK2 prevents binding of BRG1 to Rb. Phosphorylation of BRG1 by cyclin E-cdk2 inhibits its SWI/SNF chromatin remodeling activity. Further, cyclin E binds BRG1 and BAF155 of SWI/SNF complex (Shanahan et al., 1999). Without the Rb-

hSWI/SNF activity, cyclin A and cdc2 are expressed and cells enter M phase (Zhang et al., 2000).

As transcription factors like Rb recruit multiple chromatin remodeling factors, the question of timing needed to be addressed. In vitro reconstitution studies show that Gal4-VP16 transcriptional activator recruits chromatin remodeling factors (ACF) to induce chromatin remodeling at promoter, followed by HATs (e.g., CBP), which acetylate histones, resulting in displacement of H2A-H2B dimers from the surrounding nucleosomes (Ito et al., 2000). It is interesting to note that H2A-H2B dimers are labile in transcriptionally active, highly acetylated nucleosomes (Hendzel and Davie, 1990; Davie, 1995).

Transcription factors and chromatin remodeling factors are recruited to the yeast *HO* promoter in a specific order throughout the cell cycle (Cosma et al., 1999). Transcription factor Swi5p binds to the promoter soon after cells initiate anaphase and is bound to the promoter for a brief time. Once bound to the promoter, Swi5p recruits SWI/SNF. This is followed by recruitment of newly synthesized Ash1p, which puts a halt to the recruitment of SWI/SNF by Swi5p. The presence of SWI/SNF at the promoter results in the recruitment of SAGA. The histone acetylation, chromatin remodeling activity of SAGA leads to the binding of transcription factor SBF, which is required for *HO* transcription. The results of these studies show that SWI/SNF is recruited before the HAT SAGA and that histone acetylation occurs before transcription (Krebs et al., 1999; Cosma et al., 1999).

Studies with an artificial hybrid promoter, which had a Gcn4 binding site placed in the *PHO5* promoter, found that the recruitment order of SAGA and SWI/SNF was different from that reported for the *HO* promoter (Syntichaki et al., 2000). Gcn5-dependent acetylation proceeded nucleosome remodeling by SWI/SNF. The bromodomain of Gcn5 had a role in stabilizing SWI/SNF interaction with the promoter. These studies suggest that order of

recruitment and function of the chromatin modifying/remodeling factors will be promoter specific.

Also recruited to the transcriptionally competent/active chromatin sites are the HDAC complexes. HDAC activity at these loci is poised to erase the acetylation signature of active genes (Krebs et al., 1999; Wittschieben et al., 2000). The constant presence of SWI/SNF at specific yeast gene loci (e.g., *HO* promoter) may be to overcome the repression by Sin3/Rpd3 (Sudarsanam and Winston, 2000).

The balance of the HAT and HDAC activities will decide the accessibility of the chromatin to transcriptional activators and transcription machinery. The hormone receptors are excellent examples of transcription factors that can alter the chromatin micro-environment. When bound to ligand, the estrogen receptor is rapidly recruited to nuclear matrix sites (Stenoien et al., 2000) (Fig. 2). The ligand, however, decides the mix of chromatin remodeling complexes that will be recruited to estrogen-responsive genes. HATs such as CBP, PCAF, SRC-1 are recruited to estradiol bound estrogen receptor. SWI/SNF and DRIP are also recruited (Burakov et al., 2000). The temporal sequence of the recruitment of these chromatin remodeling complexes is not known. Jointly, these chromatin remodeling activities result in acetylation of estrogen responsive gene promoters and the formation a chromatin environment that will support transcription (Chen et al., 1999). However, when hydroxytamoxifen is bound to estrogen receptor, the Sin3 complex is recruited, establishing a repressive chromatin environment.

Like ligand-induced structural switching of hormone receptors, phosphorylation of transcription factors may alter their association with chromatin remodeling factors. NF- κ B for example, will recruit CBP when phosphorylated, but when unphosphorylated the transcription factor recruits HDAC1 (Zhong et al., 1998; Ghosh, 1999).

X. HISTONE H3 PHOSPHORYLATION AND ACETYLATION

H3 is phosphorylated on Ser residues in its N terminal domain (Fig. 1). Phosphorylation of H3 has a role in the transcriptional activity of immediate-early response genes. Activation of the Ras-mitogen activated protein kinase (MAPK) pathway results in the rapid phosphorylation of H3 and the transcriptional activation of the early response genes *c-fos* and *c-jun*. Through application of the ChIPs assay, we provided the first direct evidence that the newly phosphorylated H3 is associated with induced *c-fos* and *c-myc* genes (Chadee et al., 1999). Subsequent studies with antibodies recognising dimodified H3 (phosphoSer10, acetylLys14) demonstrated that rapidly following stimulation of the Ras-MAPK pathway, H3 associated with the early response genes (*c-fos* and *c-jun*) becomes dimodified (Clayton et al., 2000; Cheung et al., 2000b). Phosphorylation of H3 was found to precede acetylation at lysine 14 (Lo et al., 2000; Cheung et al., 2000b).

An investigation of the substrate preference of HATs for unmodified or modified H3 demonstrated that H3 HATs (Gcn5, SAGA, ADA, CBP, p300, PCAF) preferred to acetylate the phosphorylated H3 (Lo et al., 2000; Cheung et al., 2000b). Computer modeling revealed that Arg164 of Gcn5 was positioned to interact with the oxygen molecules of phosphoSer of H3. Mutation of this residue negated the enzymes preference for phosphoH3 without altering the enzyme's HAT activity. Interestingly, yeast mutant Gcn5 (R164A) lowered the activities of a subset of Gcn5-dependent promoters (e.g., *HO* and *His3*). Importantly, the activities of these same promoters were reduced when H3 was mutated (S10A). These results provide direct evidence that H3 phosphorylation is linked to gene activation.

Two kinases have been reported to be responsible for mitogen-induced phosphorylation of H3, Rsk2 and Msk1. Coffin-Lowry patients have a mutation in the Rsk2 gene. Fibroblasts from these patients do not exhibit EGF- or TPA-stimulated phosphorylation of H3 and,

interestingly, growth factor-induced expression of the *c-fos* gene is severely impaired (Wei et al., 1999). MSK1, but neither ERKs nor Rsk2, is inhibited by H89, a protein kinase inhibitor. H89 inhibits TPA- and EGF-stimulated H3 phosphorylation and expression of *c-fos* and *c-jun* (Thomson et al., 1999). Further studies are required to decide whether either Rsk2 or Msk1, both or perhaps other kinases are mitogen-stimulated H3 kinases.

The *c-fos* gene is transcribed in quiescent cells; however, elongation of the gene is blocked approximately 100 nucleotides from the site of initiation. Stimulation of the Ras-MAPK pathway results in the release of this block in elongation. Activation of the MAPK signaling pathway results in the phosphorylation and activation of transcription factors, such as the Ets transcription factor family (Wasylyk et al., 1998). The *c-fos* promoter serum response element (SRE) is continuously occupied by SRF and Ets proteins of the TCF family; both of these factors are targets of signaling pathways (Wasylyk et al., 1998). p62 TCF and Elk-1 (members of Ets family) are direct targets of the Ras-MAPK signaling pathway and are phosphorylated by MAPK (p42/p44; also named ERK1,2) (Ramirez et al., 1997; Gille et al., 1996). CBP binds to both Elk-1 and SRF (Ramirez et al., 1997). The finding that H3 is phosphorylated before it is acetylated at lysine 14 suggests that either Rsk2 or Msk1 is recruited to the *c-fos* chromatin first followed by the HAT acetylating lysine 14 (e.g., CBP). However, acetylation of H3 at other sites or acetylation of the other core histones may occur before phosphorylation. CBP, for example, is poised to acetylate the histones before the elongation block is released. ChIP analyses of the *c-fos* chromatin will be required to resolve these issues. The phosphorylation and acetylation of H3 and perhaps acetylation of the other core histones are likely involved in release of the elongation block, by allowing the chromatin fiber to become less compact. Consistent with this hypothesis, the *c-fos* chromatin becomes more DNAase I sensitive following activation of the Ras-MAPK pathway (Feng and Villeponteau, 1992). As the H3 tail contributes to the folding

and inter-association of chromatin fibers, modification of the H3 tail by acetylation and phosphorylation may destabilize higher order compaction of the chromatin fiber.

XI. CONCLUDING REMARKS

Much has been learned about the role of chromatin remodeling complexes in altering the structure of chromatin for both transcription activation and repression. Studies show that transcriptional activators (e.g., estrogen receptor) recruit multiple chromatin remodeling complexes (Fig. 2). Using estrogen responsive genes as an example, it is important that we determine which complexes are recruited to a specific estrogen responsive promoter and the timing of their recruitment. ChIP assays with antibodies to a component of the chromatin modifying/remodeling complex will be an approach to experimentally address this problem (Cosma et al., 1999). Also important is to decide whether the recruited chromatin modifying/remodeling complex is functional (Syntichaki et al., 2000). We need to know the influence of the chromatin micro-environment of the estrogen responsive promoter and the role of the signal transduction pathways (e.g., the Ras-MAPK pathway) on the recruiting process. For each promoter, its chromatin microenvironment, transcriptional activator and coactivator requirements, and its requirements for one or more of the chromatin remodeling factors are likely to differ. Further, there are different tiers of chromatin structural alterations. Factors responsible for establishing and maintaining DNAase I sensitive domains may differ from those of preparing a transcriptionally competent promoter.

The array of chromatin remodeling factors will become more complex. Enzymes, for example, involved in histone methylation, ubiquitination and ADP-ribosylation will have roles in transcriptional activation (Strahl and Allis, 2000). Studies are in progress to identify the

enzymes involved in these processes and how they are recruited to specific gene loci. For example, there are exciting developments in the role of histone methylation in gene expression (Strahl et al., 1999; Rea et al., 2000). Another aspect of gene activation that is being pursued is the positioning of the gene, transcription factors and chromatin remodeling complexes in three-dimensional nuclear space. As one of my colleagues recently commented, it is an exciting time to be a researcher in chromatin.

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FIGURE LEGENDS

Fig. 1. Sites of post-synthetic modifications on the core histones. The structures of the core histones H2A, H2B, H3, and H4 and the sites of modification are shown. The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and methylation (Me). The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; PP1, protein phosphatase).

Fig. 2. Recruitment of coactivators/HATs and corepressor/HDACs by nuclear matrix bound estrogen receptor. The estrogen receptor is shown associated with the nuclear matrix and its estrogen response element is in a nucleosome. When bound to estradiol, the estrogen receptor will recruit coactivator/HATs and chromatin remodeling complexes, resulting in acetylation of histones, remodeling of chromatin, and activation of transcription. But when bound to hydroxytamoxifen, estrogen receptor will recruit corepressors/HDACs, resulting in histone deacetylation and gene repression. PIC, preinitiation complex.

Fig. 3. HDAC-interacting proteins characterized to date that are involved in transcriptional repression. HDAC1 and HDAC2, together with RbAp46/48, are components of two large multiprotein complexes (Sin3 and NuRD) containing mSin3A/B or CHD3/CHD4, respectively. Note that some proteins associate with HDAC and/or HDAC binding proteins only in certain cell types. Direct binding between proteins is indicated by arrows. MEF, myocyte enhancer factor 2; * denotes receptors are bound to their respective antagonist.

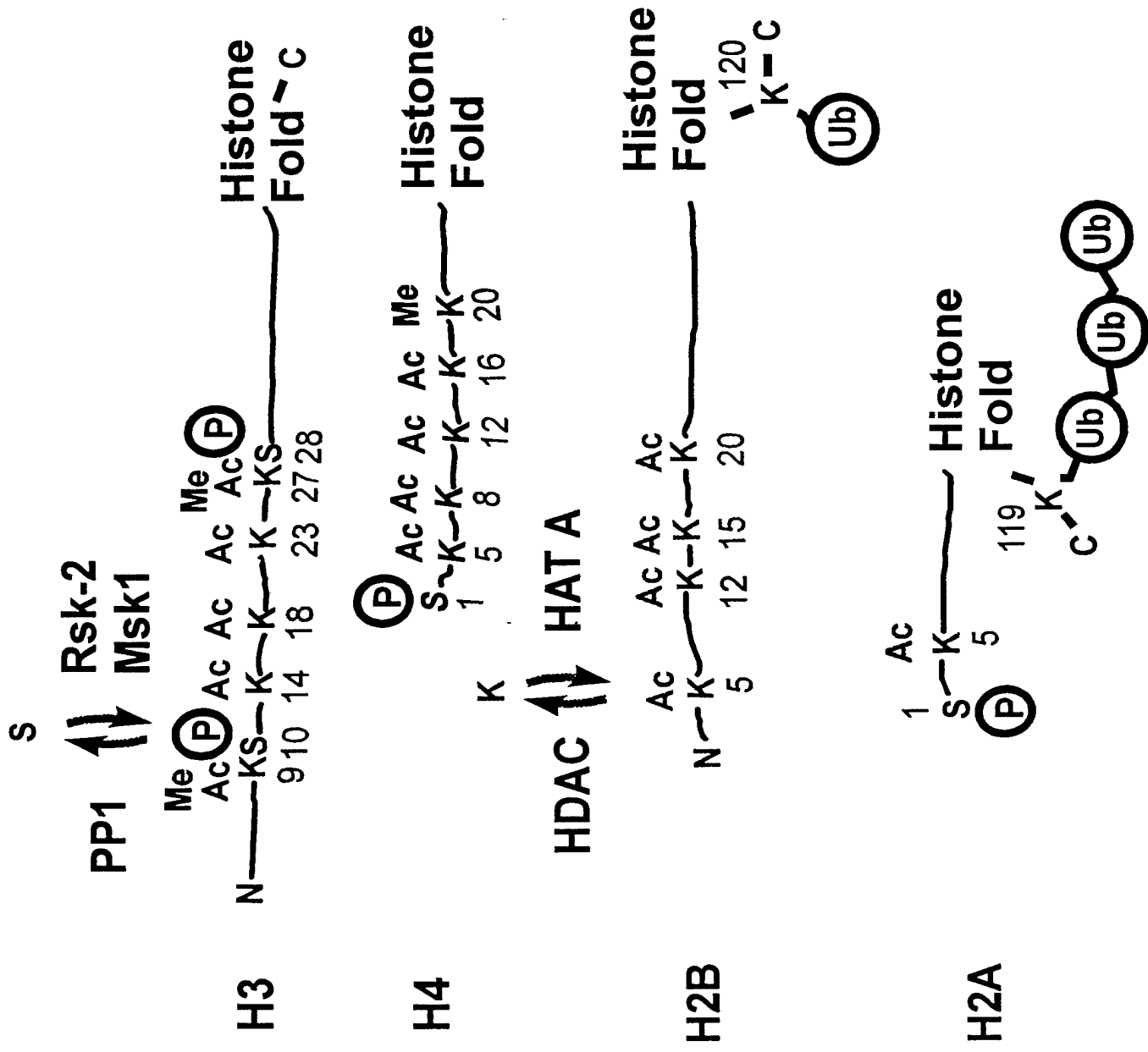


Figure 1

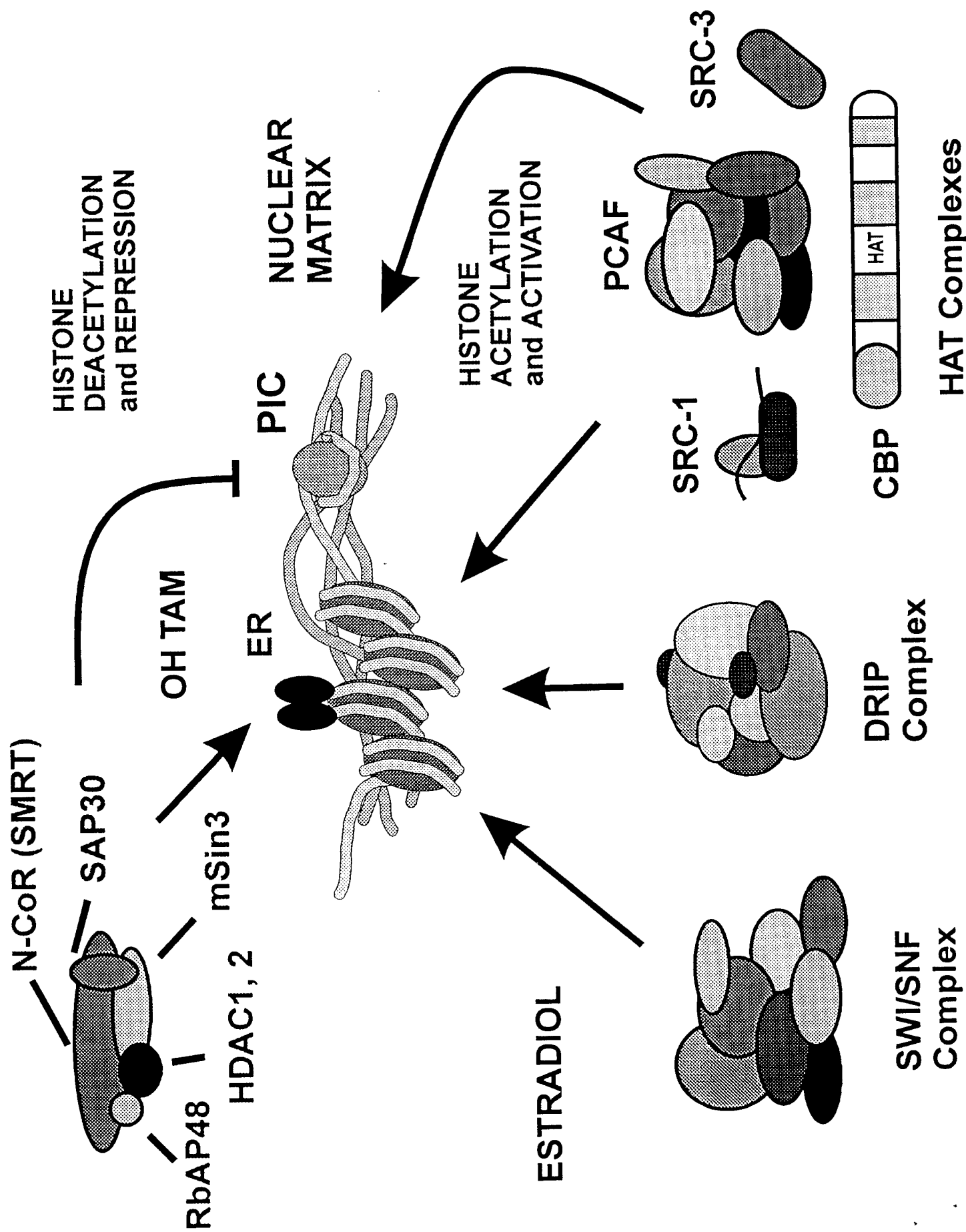


Figure 2

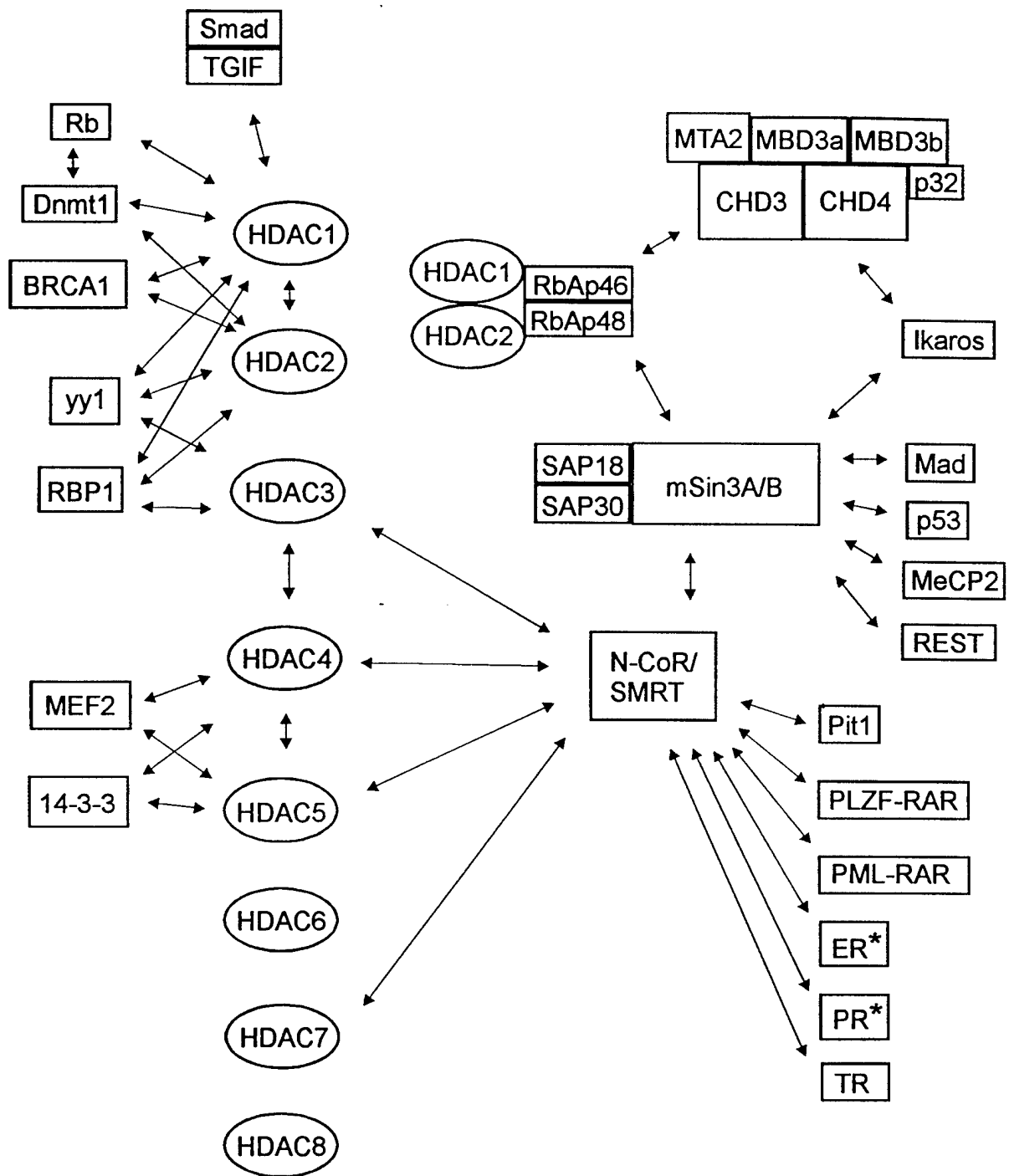


Figure 3

Signal Transduction Pathways and the Modification of Chromatin Structure

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Mechanical and chemical signaling pathways are involved in transmitting information from the exterior of a cell to its chromatin. The mechanical signaling pathway consists of a tissue matrix system that links together the three-dimensional skeletal networks, the extracellular matrix, cytoskeleton, and karyoskeleton. The tissue matrix system governs cell and nuclear shape and forms a structural and functional connection between the cell periphery and chromatin. Further, this mechanical signaling pathway has a role in controlling cell cycle progression and gene expression. Chemical signaling pathways such as the Ras/mitogen-activated protein kinase (MAPK) pathway can stimulate the activity of kinases that modify transcription factors, nonhistone chromosomal proteins, and

histones. Activation of the Ras/MAPK pathway results in the alteration of chromatin structure and gene expression. The tissue matrix and chemical signaling pathways are not independent and one signaling pathway can affect the other. In this chapter, we will review chromatin organization, histone variants and modifications, and the impact that signaling pathways have on chromatin structure and function. © 2000 Academic Press

I. Chromatin Organization

Nuclear DNA exists as a hierarchy of chromatin structures, resulting in compaction of the nuclear DNA about 10,000-fold. The histones provide an essential role in the compaction of nuclear DNA. Eukaryotes have five classes of histones, H2A, H2B, H3, H4, and H1. H2A, H2B, H3, and H4 are referred to as the core histones. The core histones are arranged as an octamer in the nucleosome core particle, the basic repeating structural unit in chromatin. The core histone octamer is organized as an (H3-H4)₂ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. Around the histone octamer core are wrapped 146 bp of DNA, forming the nucleosome core particle. The crystal structure of the nucleosome core particle was reported in 1997 (1).

The core histones (H2A, H2B, H3, and H4) have a similar structure, with a basic N-terminal domain, a globular domain organized by the histone fold, and a C-terminal tail (Fig. 1). The distribution of basic amino acids in the core histones is asymmetric, with the N-terminal portion of the molecule having a high amount of the basic amino acid residues. Some core histones (e.g., H3 and H2A) also have a short C-terminal tail. H3 and H4 are evolutionarily conserved, as is the histone fold portion of the H2A and H2B. The histone fold domains of the four core histones mediate histone-histone and histone-DNA interactions (1).

The nucleosomes are joined by linker DNA, which is of varying length. The H1 histones or linker histones bind to the linker DNA and to core histones. H1 stabilizes the higher order compaction of chromatin. H1 has a tripartite structure consisting of a central globular core and lysine-rich N- and C-terminal domains (Fig. 2). The globular domain site I, which has a winged helix DNA-binding domain found in the hepatocyte transcription factor HNF3 (2), binds to one linker DNA strand as it exits or enters the nucleosome, whereas the globular domain site II, which consists of basic amino acids, binds to nucleosomal DNA near the dyad axis of symmetry of the nucleosome (3). Not all H1 histones have the globular domain. *Tetrahymena thermophila* macronuclear H1, for example, lacks a globular domain. In contrast, yeast H1 (Hho1p) has two globular domains.

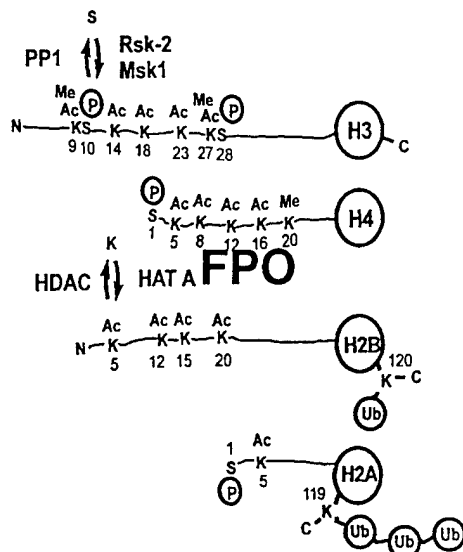


FIG. 1. Sites of postsynthetic modifications on the core histones. The structures of the H2A–H2B dimers, the (H3–H4)₂ tetramers, H1b, and the sites of modification are shown. The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and methylation (Me). The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; PP1, protein phosphatase).

Chromatin in the interphase nucleus is highly compact and is organized into chromosome territories (4, 5). Decondensation of the chromatin fiber to the 30-nm form, the structure of which remains controversial (2), is rarely observed in the interphase nucleus (4, 6). Chromosome territories are visualized by fluorescent *in situ* hybridization with chromosome-specific DNA whole library probes. However, a novel method to monitor chromosome territories

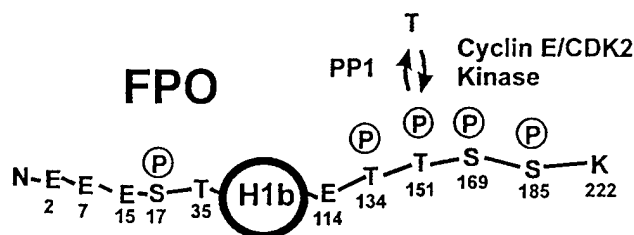


FIG. 2. Sites of postsynthetic modifications on mouse histone H1b. The sites of phosphorylation (P) and the enzymes catalyzing reversible phosphorylation (Cdk2, cyclin-dependent protein kinase 2; PP1, protein phosphatase) are shown.

and their dynamics has been developed by Cook and colleagues. By incorporating a fluorescent analog of thymidine triphosphate into nuclear DNA, direct imaging of chromatin in living cells was made possible (7).

II. Core Histone Tails

The N-terminal tails of the core histones emerge from the core particle in all directions (1). The lengths of the N-terminal tail domains vary from 16 to 44 amino acids (H3, 44 amino acids; H4, 26 amino acids; H2B, 32 amino acids; H2A, 16 amino acids) (1). Removal of the histone tails by protease digestion does not affect the structural integrity of the nucleosome. Thus, the core histone tails are not required to maintain nucleosome structure. However, removal of the N-terminal tails prevents the chromatin fiber from establishing a stable 30-nm fiber in the presence of H1 histones, showing that the tails are essential for condensation of chromatin. Genetic studies in yeast have revealed the importance of core histone tails in repression (8) and of individual tails in repression or activation of specific genes (9). The core histone tails have been thought to lack structure. However, it has been reported that in the nucleosome core particle, half of the residues in the H3 and H4 tails adopt an α -helical structure when bound to nucleosomal DNA (10). It has been proposed that the N-terminal tails undergo an induced folding when in contact with other proteins or DNA (11).

In vitro chromatin structural studies have identified a spectrum of chromatin structural states, including unfolded, moderately folded, and extensively folded conformations (12, 13). The histone tails are involved in the genesis of these chromatin structural states. In the absence of H1, the H3 and H4 tails are needed for the formation of the moderately folded chromatin conformation, whereas all core histone tails are required to mediate extensive chromatin folding at physiological ionic strength. Either H2A and H2B tails or H3 and H4 tails are needed for interfiber interactions, which result in oligomerization, to occur at physiological ionic strength (14, 15).

At low ionic strength the chromatin fibers appear as irregular, three-dimensional structures (13). The globular domain of H1 and either the H1 tails or the H3 tail domain are needed to stabilize this three-dimensional arrangement of nucleosomes (13). The other core histone tails cannot ~~be~~ substitute for the H3 tail. It is thought that the length of the H3 tails and their position of exit from the nucleosome enable the H3 tails to contribute to the three-dimensional structure of chromatin. The exit of H3 tails from the nucleosome near the linker DNA entry-exit points would position the H3 tail to make interactions with the linker DNA (13).

The core histone N-terminal tails are available for interaction with other histones and nonhistone chromosomal proteins. Richmond and colleagues

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observed that the H4 tail (K16 to N25) binds to the H2A–H2B dimer of a neighboring nucleosome. This interaction would contribute to the folding of the chromatin fiber, and it may be involved in nucleosome positioning (1, 8). This region of yeast H4 also has an important role in telomeric silencing (9).

Nonhistone chromosomal proteins interact with the tails of H3 and/or H4 to form a transcriptionally competent or repressive chromatin structure. In yeast, the H3 and H4 tails bind to the trans-acting repressors, Sir3 and Sir4, leading to the formation of a transcriptionally repressed chromatin domain (16, 17). The N-terminal tails of yeast H3 and H4 also bind to the global repressor Ssn6/Tup1 (18). *Drosophila* Groucho and its mammalian homologs, the transducin-like Enhancer of split proteins, are transcriptional repressors that bind to the N-terminal tail of H3 (19, 20). High-mobility-group¹ (HMG)-14 and -17 proteins bind to nucleosomes and unfold the higher order chromatin fiber, facilitating transcription (21). The C-terminal domain of HMG-14, which is involved in chromatin unfolding, binds to the N-terminal tail of H3 (amino acid residues 20 to 50) (21).

III. Transcriptionally Active Chromatin

Transcriptionally active DNA is associated with nucleosomes. However, nucleosome structure is perturbed on transcription with RNA polymerase II

¹ Abbreviations: HMG, high mobility group; DNase I, deoxyribonuclease I; IGC, interchromatin granule cluster; HAT, histone acetyltransferase; CBP, CREB (cAMP response element binding protein)-binding protein; PCAF, p300/CBP-associated factor; Esa1, essential SAS2-related acetyltransferase; Elp3, elongator protein 3; SAGA, Spt-Ada-Gcn5-acetyltransferase; TAF_{II}, RNA polymerase II-specific TAT-binding protein-associated factor; TRRAP, human transformation/transcription domain-associated protein; Tip60, Tat-interacting protein 60; MORF, monocytic leukemia zinc finger protein-related factor; MOZ, monocytic leukemia zinc finger protein; SRC-1, steroid receptor coactivator 1; RAC3, receptor-associated coactivator 3; AIB1, amplified in breast cancer 1; TRAM-1, thyroid hormone receptor activator molecule 1; MAPK, mitogen-activated protein kinase; HDAC, histone deacetylase; NuRD, nucleosome-remodeling histone deacetylase complex; SAP, Sin3-associated polypeptide; Rb, retinoblastoma protein; RbAP, Rb-associated protein; N-CoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; MBD, methyl-CpG-binding domain-containing protein; RBP1, Rb-binding protein 1; ER, estrogen receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor; MEF2, myocyte enhancer factor 2; MITR, MEF-2 interacting transcription repressor; PML, promyelocytic leukemia; PLZF, promyelocytic leukemia zinc finger; BTB/POZ, bric-à-brac tramtrack broad complex/pox viruses and zinc fingers; LAZ3/BCL6, lymphoma-associated zinc finger 3/B cell lymphoma 6; CHIP, chromatin immunoprecipitation; CDK, cyclin-dependent kinase; SRE, serum response element; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; S/MARs, scaffold/matrix-attached regions; NMTS, nuclear matrix targeting sequence; GFP, green fluorescent protein; ARBP, attachment region binding protein.

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(22, 23). Nucleosomes associated with transcribed chromatin have an unfolded structure, exposing a normally buried cysteine at position 110 in H3 (24, 25). Nucleosomes associated with newly replicated chromatin do not have exposed H3 thiol groups. Transcriptional elongation is required to form the unfolded nucleosome (23). Nucleosomes associated with RNA polymerase II-transcribed genes in mammalian cells cease to be thiol reactive when RNA polymerase II transcription is arrested with α -amanitin. Garrard and colleagues reported that the process of transcription did not cause disruption of chromatin structure, because transcription by T7 RNA polymerase did not disrupt nucleosome structure (22). Why transcription by RNA polymerase II, but not T7 RNA polymerase, perturbs chromatin structure is not clear. However, possible factors include the duration of torsional stress induced by RNA polymerase II transcription (22) and that the process of RNA polymerase II transcription occurs at the karyoskeleton (26, 27).

The chromatin of transcriptionally active genes differs from the bulk of the genome in susceptibility to digestion by nucleases, including micrococcal nuclease and deoxyribonuclease I (DNase I) (28). The nucleosome disruption is largely confined to the DNA sequences of the transcribed region. However, the preferential DNase I sensitivity of active genes is not restricted to the coding portion of the gene but extends far upstream and downstream into adjacent nontranscribed DNA sequences before converting to a DNase I-resistant conformation. Typically the DNase I sensitivity of transcriptionally active domains is 2- to 3-fold greater than that of DNase I-resistant chromatin. However, in one study of the β -globin gene domain in 12-day embryonic red blood cells, a DNase I sensitivity of 20-fold was observed. This increase in DNase I sensitivity is due to the preservation of torsional stress within the β -globin domain. Nicking the DNA within the domain by γ rays causes a reversal of DNase I sensitivity from 20-fold to 2- to 3-fold (29). These studies found that a single nick in the DNA of the domain was sufficient to dissipate the torsional stress within the domain.

Extended chromatin loops with decondensed (30-nm fiber) regions, which are presumably transcribed, have been observed in G_1 phase nuclei of Chinese hamster ovary cells (4). Several studies show that sites of transcription are found at or near the surface of compact chromosome subdomains (5, 30). Interestingly, these decondensed chromatin regions are often found near interchromatin granule clusters (IGCs), ribonucleoprotein structures that serve as storage sites for splicing factors (6, 30). Highly transcribed genes are located near IGCs (30), and highly, dynamically acetylated histones are associated with the chromatin positioned near these structures (6).

IV. Histone Variants and Modifications

Histone variants and particularly their modified isoforms modify higher order chromatin packaging and are tightly linked to the transcription process. In the following sections, histone variants and modifications are reviewed.

A. Core Histone Variants

Variants of the core histones generate considerable complexity in the histone octamers of the nucleosomes, impacting on chromatin structure. There are multiple forms of H3, H2A, and H2B, which have microheterogeneity in their primary sequence. For example, in the DT40 chicken B cell line there are three variants of H2A, two variants of H3, and four variants of H2B (31). The population of nucleosome histone variants changes during development, differentiation, and lymphocyte activation (32). There is evidence that the histone variants may be of importance structurally and functionally (31, 33). Deletion of an H2B variant in DT40 cells results in changes in the cellular protein profile, suggesting gene-specific effects (34).

The amino acid sequence of some histone variants can differ markedly from the major histone. H2A.Z, a member of the histone H2A family, is evolutionarily conserved and appears to be a component of transcriptionally active chromatin (35). The amino acid sequences of histone H2A.Z. and H2A differ by 40%. Deletion of the *Drosophila* gene coding for the histone variant H2AvD, which is similar to mammalian H2A.Z, is lethal. Similarly, expression of the H2A.Z gene in *T. thermophila* and mice is required for viability (33). MacroH2A is a novel histone H2A variant that is expressed in mammalian and avian cells (36). The N-terminal third of macroH2A is 64% identical to H2A, whereas the remainder of the protein has a segment that resembles a leucine zipper, a dimerization motif found in many transcription factors. In female mammalian cells, macroH2A is preferentially located with the inactive chromosome (37). Cse4P and CENP-A are H3 variants that are found in centromeric chromatin of *Saccharomyces cerevisiae* and mammals, respectively (38, 39). The N-terminal domains of these H3 variants are unique, but the C-terminal histone folds of Cse4P and CENP-A are about 60% identical to the histone-fold domain of H3.

B. Histone H1 Subtypes

The H1 histones are a heterogeneous group of several subtypes that differ in amino acid sequence. The H1 subtypes differ in their abilities to condense DNA and chromatin fragments. Thus, the differential distribution of the H1 histones with chromatin domains may generate chromatin regions with different degrees of compaction (28). Most nuclei typically have more than one H1 subtype. However, there are exceptions. Trout testis, for example,

has one H1 subtype. The relative amounts of the H1 subtypes vary with cell type within a particular species, as well as among various species. For example, mouse tissues contain various levels of H1 subtypes, H1a, H1b, H1c, H1d, H1e, and H1^o. A nomenclature for the mammalian H1 histones has been proposed (40). The expression of the subtypes is differentially regulated throughout development, through the cell cycle, and during differentiation (41). Changes in the expression of H1 subtypes affect gene expression (41, 42).

C. Core Histone Modifications

The ability of the core histones to promote chromatin intrafiber and interfiber interactions is modulated by histone modifications. The core histone tails are susceptible to a wide range of postsynthetic modifications, including acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP-ribosylation (Fig. 1). We have known about these modifications since the 1960s, but it has only been recently that we have come to appreciate the impact of these modifications on nuclear processes such as transcription. Most modifications occur on the N-terminal basic tail domain, with histone ubiquitination being the exception. In the following sections we will review histone ubiquitination, acetylation, methylation, and phosphorylation and their roles in gene expression.

D. Histone Ubiquitination

Histones H2A, H2B, H3 and their variant forms are reversibly ubiquitinated (28). The carboxyl end of ubiquitin, a highly conserved 76-amino acid protein, is attached to the ϵ -amino group of lysine (K119 in H2A; K120 in H2B) (Fig. 1). The linkage of ubiquitin to H3, which has only been observed in elongating spermatids of rat testis, is not known (43). In multicellular eukaryotes, H2A is typically ubiquitinated to a greater extent than H2B (approximately 10% of H2A versus about 1–2% of H2B). H2A, H2B, and their variants are also polyubiquitinated, with H2A having the greater levels of polyubiquitinated isoforms. The major arrangement of ubiquitin in polyubiquitinated H2A is a chain of ubiquitin molecules joined to each other by isopeptide bonds to a ubiquitin molecule that is attached to the ϵ -amino group of K119 of H2A (28).

Ubiquitinated H2B and to a lesser extent ubiquitinated H2A are associated with transcriptionally active DNA. Ubiquitination of H2B is the only core histone modification that is dependent on ongoing transcription (44). The C-terminal sequence of H2B, but not H2A, is buried in the nucleosome (1). It is thought that the process of transcription disrupts nucleosome structure, exposing the C terminus of H2B to become accessible to the enzymes catalyzing the addition of ubiquitin (22, 44). Another mechanism, which

does not require ongoing transcription, is by exchange of newly synthesized ubiquitinated H2B and H2A with histones that were in transcriptionally active nucleosomes of G₀ phase cells (45). The introduction of ubiquitinated H2B into the nucleosome may result in an alteration in nucleosome and/or higher order chromatin structure. In H2B the tyrosine residue positioned next to the site of ubiquitination interacts with H2A N-terminal tail (residues 17 to 20) just before it exits the nucleosome (1). H2B ubiquitination would likely interfere with this interaction.

E. Histone Acetylation

The core histones are reversibly acetylated at specific lysine residues located in the N-terminal tail domains. With the exception of H2A, the core histones are acetylated at four to five sites. Thus, a nucleosome typically has 26 sites of acetylation. Over the past few years, the role of histone acetylation in the process of transcription has been solidified (46).

Histone acetylation is a dynamic process that is governed by the net activity of histone acetyltransferases and histone deacetylases. The process of reversible histone acetylation is not dependent on ongoing transcription. Acetylation occurs at more than one rate, as does the subsequent deacetylation. In mammalian cells and mature avian erythrocytes, one population of core histones is characterized by rapid hyperacetylation ($t_{1/2} = 7$ to 15 min for monoacetylated histone H4) and rapid deacetylation ($t_{1/2} = 3$ to 7 min) (47–49). This highly dynamic acetylation–deacetylation is limited to 15% (hepatoma tissue culture cells) of the core histones (47). A second population is acetylated ($t_{1/2} = 140$ to 300 min for monoacetylated H4) and deacetylated at a slower rate ($t_{1/2} = 30$ min) (47–49).

Approximately 2% of the chromatin of adult chicken immature erythrocytes has core histones that participate in active acetylation and deacetylation. The bulk of the chicken erythrocyte chromatin has core histones frozen in unacetylated and monoacetylated states. There is one rate of acetylation for the immature erythrocyte dynamically acetylated histones ($t_{1/2} = 12$ min for monoacetylated H4), which is in contrast to two rates of acetylation in mature cells (49). One population of dynamically acetylated H4 is rapidly acetylated to the mono- and diacetylated states, and is slowly deacetylated. Another population of dynamically acetylated H4 is rapidly acetylated to the tetraacetylated state, and the tetraacetylated H4 isoform is rapidly deacetylated ($t_{1/2} = 5$ min). Thus, the chicken erythroid tetraacetylated H4 isoform is short lived. Of the four core histones, H2B is the most rapidly deacetylated (49, 50). The rates of deacetylation of monoacetylated H4 are also different in adult chicken mature and immature erythrocytes (145 versus 90 min, respectively) (50). In chicken immature erythroid cells, the dynamically highly acetylated histones appear to be associated with transcriptionally active

chromatin, including transcribed chromatin associated with the karyoskeleton (49–52).

In yeast *S. cerevisiae* there is a high level of highly acetylated core histones (53). These highly acetylated histones are slowly acetylated and deacetylated (53). Regions of the yeast genome that are transcriptionally silent are associated with hypoacetylated histones (54). In contrast to yeast, approximately 20% of the chromatin of the unicellular green alga *Chlamydomonas reinhardtii* has core histones that are dynamically multiacetylated with a half-life of about 2 min (55).

Alterations at all levels of chromatin structure are invoked by acetylation of the core histones. Acetylation of the histone tails disrupts higher order chromatin folding (56), promotes the solubility of chromatin at physiological ionic strength, and maintains the unfolded structure of the transcribed nucleosome (57). Analysis of chromatin fibers enriched in transcribed genes and acetylated histones revealed that these chromatin fibers underwent compaction but not oligomerization as the concentration of NaCl was raised to 150 mM. Additional studies show that nucleosomes do not have to be maximally acetylated to prevent higher order chromatin folding. Hansen and colleagues demonstrated that acetylation to 46% of maximal site occupancy was sufficient to prevent higher order folding and stimulation of transcription by RNA polymerase III (58). It has been proposed that acetylation of core histone tails interferes with folding of the N-terminal tail and interactions with proteins and/or DNA, destabilizing higher order chromatin organization (11, 56). These combined effects of histone acetylation on the destabilization of chromatin structure facilitate transcription (58, 59).

Histone acetylation can affect the interaction of nonhistone chromosomal proteins with chromatin in at least two ways. First, histone acetylation facilitates the interaction of transcription factors with nucleosomal DNA (60). Partial acetylation of the core histone tails is sufficient to expose nucleosomal DNA for transcription factor binding without displacement of the N-terminal tail domains from DNA (61). Second, for proteins that interact with the N-terminal tail domain, acetylation may modulate these interactions. For example, acetylation disrupts interactions between the tail domain and the repressor Tup1 (18).

1. HISTONE ACETYLTRANSFERASES AND GENE ACTIVATION

The first histone acetyltransferase (HAT) gene cloned (*Tetrahymena* nuclear HAT p55) was found to be homologous to yeast Gcn5, a transcriptional adapter/coactivator with HAT activity (62). This pivotal discovery told us how HATs were directed to transcribed chromatin regions. Following the discovery that the transcriptional activator, Gcn5, had HAT activity, many other coactivators with HAT activity have since been identified, including p300/CBP-associated factor (PCAF), CBP/p300, Esa1, NuA4, steroid receptor

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TABLE I
HISTONE ACETYLTRANSFERASES AND THEIR SUBSTRATES^a

HAT A (organism; proteins in complex)	Free Histone or other substrate	Nucleosomal histone substrate
Gcn5 (yeast, human, <i>Drosophila</i>)	H3 > H4 (K14 of H3; K8, 16 of H4)	—
Ada (yeast; Gcn5 and Ada proteins)	—	H3, H2B
SAGA (yeast; Gcn5, Ada, and Spt proteins, TAF _{II} s, and Tra-1, a homolog of the TRRAP)	—	H3 > H2B
STAGA (human; Gcn5-L, Spt3, TAF _{II} 31)	H3 > H4	—
TFTC (mammalian; Gcn5-L, hAda3, hSpt3, hTRAPPM TAF _{II} s)	H3	H3
PCAF (human; human counter- parts of yeast ADA proteins, Spt proteins, human TAF _{II} s, and PAF400, a 400-kDa protein almost identical to TRRAP)	H3 > H4, TFIIF, TFIIE	H3
Esal (yeast)	H4 > H3 > H2A (K5 > K8, 12, 16 of H4; K14 of H3; K5 of H2A)	—
NuA4 (yeast, Esal)	—	H4, H2A
NuA3 (yeast)	—	H3
CBPp300 (human)	H3, H4 > H2A, H2B (K5, 8, 12, 16 of H4), TFIIF, TFIIE, p53, EKLF, ACTR, SRC-1	H3, H4, H2A, H2B
TAF _{II} 250 (human), <i>Drosophila</i> , yeast)	H3 > H4 (K14 of H3), TFIIE	—
Tip60 (human)	H4 > H3 > H2A (K5 > K8, 12, 16 of H4)	—
MORF (human)	H4 > H3 >> H2B (K5 > K8, 12, 16 of H4)	H4 > H3
Elp3 (yeast, elongating RNA polymerase II holoenzyme)	H4, H3, H2A, H2B	Not known
SRC-1 (human)	H3 > H4 (K9, K14 of H3)	H3, H4, H2A, H2B
ACTR (human)	H3, H4 > H2B	H3 > H4

^aFor references, see histone acetyltransferase Internet site at <http://www.mdanderson.org/genedev/Bone/hathome.html>.

coactivators, and most recently Elp3 (63; for review see 64, 65) (Table I). We have now come to appreciate the mechanistic connections between histone-modifying activities and the RNA polymerase II machinery.

The solution and crystal structures of the HAT domain of *Tetrahymena* Gcn5, yeast Gcn5, and PCAF have been reported (66–69). Glu-173 in yeast Gcn5, Glu-122 in *Tetrahymena* Gcn5, and Glu-570 in PCAF are essential

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residues in catalysis (66, 69, 70). It is thought that the structural and functional properties of the catalytic domains of other HATs will be similar. The crystal structure of another domain, the bromodomain, commonly found in HATs has been presented (71). Interestingly, this domain in Gcn5 and PCAF interacts with the N terminus of H3 and H4 and may be involved in targeting the coactivator to chromatin (71, 72). The bromodomain of human Gcn5 binds also to the DNA-dependent protein kinase. The recruited kinase phosphorylates Gcn5, inhibiting HAT activity (73).

The substrate specificities of the HATs differ. Further, many of the HATs are in multiprotein complexes, and the substrate specificity of the HAT will vary depending on whether the enzyme is free or in a complex (for review see 73). For example, yeast Gcn5 acetylates free H3, but inefficiently acetylates histones in nucleosomes. In mammalian cells, differentially spliced forms of *Gcn5* transcripts generate different Gcn5 isoforms (74). A 98-kDa-long form of mammalian Gcn5 has an N-terminal extension not found in yeast Gcn5. Although the presence of the N-terminal extension does not change the substrate specificity of Gcn5 toward free histones, it does enable the mammalian Gcn5 to acetylate nucleosomal H3 (74). The N-terminal extension is similar to that of human PCAF; both proteins bind to other coactivators with HAT activity, e.g., CBP/p300. The C-terminal domain of PCAF is similar to that of yeast Gcn5.

Yeast Gcn5 will efficiently acetylate histones in nucleosomes only when it is in high-molecular-weight multiprotein complexes such as Spt-Ada-Gcn5-acetyltransferase (SAGA) (1.8 MDa) and Ada (0.8 MDa) (75). Although both complexes contain Ada3 and Ada2, which binds to yeast Gcn5, the two complexes are distinct (76). The SAGA complex contains Ada, Spt proteins [Spt20 (Ada5), 3, 7, and 8], TAF_{II}s (TAF_{II} 90, 68/61, 60, 25/23, 20/17), and Tra-1, a homolog of the human transformation/transcription domain-associated protein (TRRAP) (73, 77). TAF_{II}68, which is homologous to human TAF_{II}20 and related in structure and sequence to H2B, is required for the integrity, nucleosomal acetylation, and transcriptional enhancing activities of SAGA (77). Yeast TAF_{II}60 and TAF_{II}17 have sequence similarities to H3 and H4 and interact with each other as a heterotetramer through a histone fold. These observations suggest the presence of a histone octamer-like structure within the SAGA complex.

Similar to yeast SAGA, human PCAF is in large multiprotein complexes consisting of human counterparts of yeast Ada proteins, Spt proteins, human TAF_{II}s (TAF_{II} 31, 20/15, and two TAF-like proteins, PAF65 α and PAF65 β), and PAF400, a 400-kDa protein almost identical to TRRAP (73). Human TAF_{II}31 and TAF_{II}20/15 have the histone fold structure found in histones H3 and H2B, respectively. PAF65 α has similarity to human TAF_{II}80 and has an H4-like region, whereas PAF β has similarity to the WD40 repeat-containing TAF_{II}100 (78).

Esal (essential SAS2-related acetyltransferase; the *ESAL* gene is essential for yeast growth) and Tip60 (Tat-interacting protein 60) are members of the MYST family of proteins (named after founding members, MOZ, YBF2/SAS3, SAS2, and Tip60). Esal and Tip60 are HATs with similar substrate specificities (79). Neither protein can acetylate chromatin substrates. The monocytic leukemia zinc finger protein-related factor (MORF) shares significant sequence similarity with MOZ and has HAT activity (80). MORF, Tip60, and Esal share a conserved HAT domain. Unlike Esal and Tip60, MORF acetylated H4 and H3 in oligonucleosomes (80). However, when yeast Esal is assembled into a multiprotein complex called NuA4, the complex will acetylate nucleosomal H2A and H4 (see Table I) (81, 82). Similar to other HATs, NuA4 is associated with Tra1 (82). A HAT with properties similar to those of yeast NuA4 (about 1.3 MDa) was isolated from *Tetrahymena*. One difference, however, is that the *Tetrahymena* HAT (80 kDa) appears to exist as a single protein or as a small multiprotein complex (81).

CBP/p300, a coactivator with HAT activity, is an integrator of multiple signaling pathways (Fig. 3). Transcription factors, including hormone receptors, CREB, and fos-jun, loaded onto promoters or enhancers bind directly or indirectly to CBP/p300. Further, CBP is a component of the RNA polymerase II holoenzyme. RNA helicase A binds to CBP and is thought to mediate an interaction between CBP and RNA polymerase II (83). The steroid receptor coactivators SRC-1 and ACTR (and related proteins RAC3, AIB1, and TRAM-1) bind to a variety of nuclear receptors in a ligand-dependent manner. These coactivators associate with CBP/p300 and PCAF. Thus, a ligand-activated nuclear receptor could recruit multiple coactivators with HAT activity (e.g., Tip60, SRC-1, CBP, and PCAF) (84, 85) (Figs. 3 and 4).

CBP is a phosphoprotein. CBP is phosphorylated by ERK1, enhancing the HAT activity of CBP *in vitro* (86). This observation suggests that the activity of CBP may be regulated by the Ras/mitogen-activated protein kinase (MAPK) pathway (87, 88).

The acetyltransferase activity of several HATs is not limited to histones. PCAF acetylates the nonhistone chromosomal protein HMG-17 (89). Acetylation of HMG-17 reduces the protein's binding affinity to the nucleosome. CBP/p300 acetylates the four core histones in nucleosomes and a variety of transcription factors (Table I). For example, CBP acetylates p53 and GATA-1 and potentiates the activities of these transcription factors (73). CBP also acetylates other HATs (e.g., ACTR, SRC-1), which disrupts the interaction of the coactivator (ACTR) with the estrogen receptor (90).

Transcription factors may recruit one or more HATs. Transcriptional activators with an acidic activation domain (e.g., VP16) or helix-loop-helix proteins with the LDFS motif (e.g., yeast transcription factor Rtg3) recruit SAGA, resulting in localized acetylation and transcriptional stimulation of

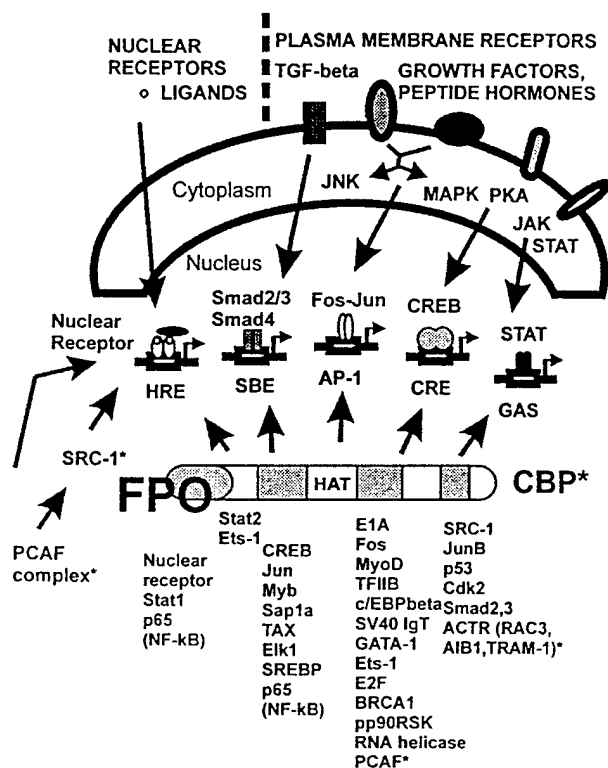


FIG. 3. The histone acetyltransferase/coactivator, CBP/p300, cointegrates diverse signaling pathways. A variety of sequence-specific transcription factors and coactivators bind to different regions of the CBP (CREB-binding protein)/p300 protein. Kinases such as JAK (Janus kinase), PKA (protein kinase A), MAPK (mitogen-activated protein kinase), and JNK (Jun amino-terminal kinase), once activated, will phosphorylate a variety of transcription factors. The signal-dependent transcription factor, once bound to its DNA binding site [e.g., HRE (hormone responsive element), CRE (CREB-responsive element), GAS (interferon-stimulated gene response element), and SBE (Smad binding element)], will recruit CBP/p300 (shown by arrows from CBP/p300). CBP/p300 and steroid receptor coactivators have histone acetyltransferase (HAT) activity (*).

nucleosomal substrates *in vivo* and *in vitro* (75, 91–93). Importantly, the transcriptional stimulatory activity of the recruited SAGA complex is dependent on its HAT activity (75). Similar to ligand-activated nuclear receptors (e.g., estrogen receptor bound to estradiol; Fig. 4), VP16 appears to recruit several HATs *in situ*, including Gcn5, PCAF, and CBP/p300 (94). NF- κ B recruits several coactivators with HAT activity, including CBP, PCAF, and SRC-1 (95). Interestingly, on phosphorylation by a cAMP-independent

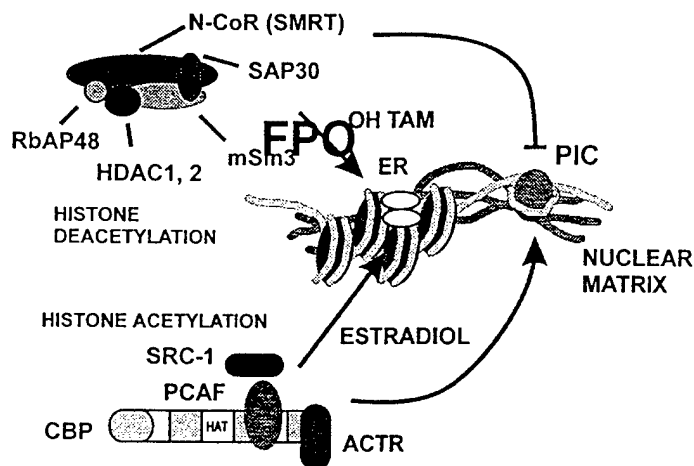


FIG. 4. Recruitment of coactivators/HATs and corepressor/HDACs by karyoskeleton-bound estrogen receptor. The estrogen receptor is shown associated with the nuclear matrix and its estrogen response element in a nucleosome. When bound to estradiol, the estrogen receptor will recruit coactivator/HATs, resulting in acetylation of histones and activation of transcription. When bound to hydroxytamoxifen, the estrogen receptor will recruit corepressors/HDACs, resulting in histone deacetylation and gene repression. PIC, Preinitiation complex.

protein kinase A, the p65 subunit of NF- κ B undergoes a conformational change that forms a bivalent interaction with CBP (96) (see Fig. 3).

2. ROLE OF HATs IN TRANSCRIPTION

Coactivator multiprotein complexes with HAT activity can stimulate transcription at several levels, including stimulating the formation of the preinitiation complex and by remodeling chromatin (73, 88, 92, 97–99) (Fig. 4). Several lines of evidence support the view that recruited coactivators with HAT activity acetylate surrounding histones in nucleosomes, leading to the destabilization of higher order chromatin structure and stimulation of transcription (65, 75, 81, 92, 100). However, it has been questioned whether the histones are the bona fide *in vivo* substrates of some HATs. The activity of some HATs may be directed toward transcription factors, affecting transcriptional processes. Studies with yeast and *Tetrahymena* HATs provide evidence that histone acetylation has a role in the transcription process. When recruited to a promoter, yeast HATs (SAGA, NuA4, NuA3, Ada) and *Tetrahymena* NuA4 facilitated transcription *in vitro* from nucleosomal, but not naked DNA, templates (75, 81, 92, 100, 101). Importantly, the HAT stimulation was observed only when acetyl CoA was present.

Acetylation of chromatin components can activate or repress transcription. The activity of the interferon- β (IFN- β) enhanceosome is regulated, in part, by acetylation. The enhanceosome consists of NF- κ B, IRF1, ATF2/c-Jun, and HMGI(Y), an essential architectural protein involved in the stereospecific assembly of this complex. Once assembled, the complex effectively recruits CBP (102), which then acetylates H3 and H4 in neighboring nucleosomes, resulting in the remodeling of chromatin and the recruitment of the RNA polymerase II holoenzyme (103). The net result is the turning on of IFN- β gene expression. However, CBP can also acetylate HMGI(Y) at a site important in DNA binding. The result of HMGI(Y) acetylation is disruption of the enhanceosome and the turning off of IFN- β gene expression (104).

3. HISTONE DEACETYLASE AND GENE REPRESSION

In 1996, the cloning of mammalian histone deacetylase 1 (HDAC1) revealed that it was related to yeast transcription regulator RPD3, providing a link between transcription regulation and histone deacetylation. Several HDACs have since been reported, including HDAC2 (the mammalian homolog of RPD3) and mammalian HDAC3 (reviewed in 46). HDACs, bacterial acetoin utilization proteins, and acetylpolymine amidohydrolases appear to be members of an ancient protein superfamily. These proteins share nine blocks of sequence similarity, with 20 amino acids being invariant in these alignments (105). Some of these conserved amino acids could be involved in binding a metal atom, e.g., zinc; there is evidence that HDAC1 is a metalloenzyme (106, 107). The crystal structure of the HDAC catalytic core based on the hyperthermophilic bacterium *Aquifex aeolicus* HDAC was reported (108). The active site consists of residues that are conserved, a zinc-binding site, and two Asp-His charge relay systems.

In contrast to HATs, recruitment of HDACs can lead to repression. It is important to note, however, that chromatin regions engaged in transcription are associated with dynamically acetylated histones (64). Thus, both HATs and HDACs are recruited to these regions. When the balance of activity of these two enzymes favors deacetylation, the chromatin region will take on a repressive higher order structure.

The HDACs have been categorized into two classes. The first class consists of yeast histone deacetylases Rpd3, HOS1, and HOS2 and mammalian HDACs, HDAC1, HDAC2 (the mammalian homolog of yeast RPD3), and HDAC3 (64). Class 2 consists of yeast HDA1 and mammalian HDAC4 (HDAC-A), HDAC5 (mHDA1, NY-CO-9, HDAC-B), and HDAC6 (mHDA2) (109–111).

Mammalian HDAC1 and HDAC2, but not HDAC3, are in large multiprotein complexes, e.g., mSin3A and NuRD (Fig. 5). The mSin3A complex contains mSin3, N-CoR or SMRT (corepressors), SAP18, SAP30, RbAp48,

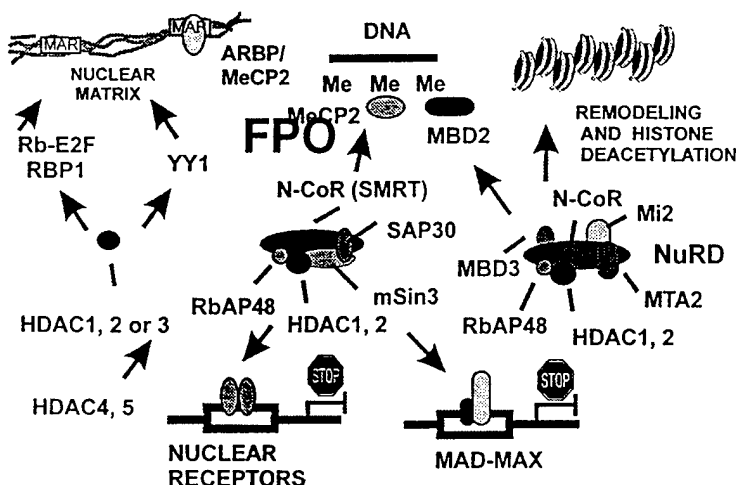


FIG. 5. The HDAC complexes are involved in several signaling pathways. The Sin3A and NuRD complexes may be recruited to specific sites by nonliganded hormone receptors to hormone response elements or by Mad-Max to E-box-related sequences. MeCP2 and MBD2 recruit the HDAC complexes to methylated DNA, resulting in silencing. ARBP/MeCP2 may also recruit the HDAC complex to scaffold/matrix-associated regions. Rb, YY1, and several other karyoskeleton-bound transcription factors could recruit HDAC complexes to the karyoskeleton.

RbAp46, and c-Ski (112). Another complex, the nucleosome remodeling histone deacetylase complex (NuRD), consists of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, RbAP46/48, and MBD3 (methyl-CpG-binding domain-containing protein), and has both ATP-dependent chromatin remodeling and HDAC activities (113). HDAC3 and the class II HDACs are not found in the mSin3A and NuRD complexes (111). However, HDAC4 and HDAC5 bind to HDAC3 (111). Like human HDAC2, yeast Sin3 and RPD3 are in a large 2-MDa protein complex. The other yeast HDACs, HDA1, HOS1, HOS2, and HOS3, are also in higher molecular mass complexes (114).

Class I and class II histone deacetylases can deacetylate the four core histones. However, *in vitro* studies show that HDACs have site preferences. For example, yeast HOS3 preferentially deacetylates yeast histones at K5 and K8 of H4, at K14 and K23 of H3, at K7 of H2A, and at K11 of H2B (114). Substrate preference is regulated by components of the multiprotein complexes. For example, free avian HDAC1 preferentially deacetylates H3, but not nucleosomal H3. HDAC1 in a multiprotein complex associated with the karyoskeleton preferentially deacetylates free H2B and will deacetylate histones in nucleosomes (107). NuRD has both ATP-dependent chromatin remodeling

and histone deacetylase activities (115–118) (Fig. 5). ATP stimulation of deacetylation of chromatin templates by NuRD varied from no stimulation to about threefold.

HDAC or HDAC complexes are recruited to specific genomic sites by transcription factors (repressors). HDACs 1, 2, and 3 bind to YY1, whereas Rb and E2F form a complex with HDAC1 (64, 65). Evidence has been presented that the IXCXE motif located in the C-terminal region of HDAC1 and HDAC2 associates with the Rb “pocket” domain of hypophosphorylated Rb, p107, and p130 (119, 120). More recent studies, however, provide evidence that binding of HDACs 1, 2, and 3 to the pocket proteins (Rb, p107, p130) requires an intermediary protein, RBP1 (121). The recruitment of the E2F–Rb–HDAC1 complex is partly responsible for the repression of the cyclin E promoter in G₁ phase of the cell cycle.

Phosphorylation of Rb by CDK4 and CDK6 results in the dissociation of Rb from HDAC1 and E2F (122). The promoter-associated E2F may now associate with CBP/p300, recruiting a HAT activity that stimulates transcription of the cyclin E gene. HDAC1 can also bind to the C-terminal domain of Sp1, repressing this transcription factor’s activity (123). E2F1 binds to the C-terminal domain of Sp1 and displaces HDAC1. Binding sites for Sp1 and E2F1 are found on S phase-specific promoters. Thus, there is an interesting relationship between Sp1, E2F, pocket proteins, and histone deacetylases in the repression of these growth-regulated genes (123).

The methyl-CpG-binding protein 2 (MeCP2) recruits the mSin3A complex, and methyl-CpG-binding domain-containing protein (MBD2) binds to the NuRD complex, providing mechanisms for coupling DNA methylation and histone deacetylation in gene silencing (113, 124). Interestingly, mutations in the X-linked gene encoding MeCP2 were found to be the cause of some cases of Rett syndrome, a progressive neurodevelopmental disorder (125).

Several signal transduction pathways regulate the recruitment of the HDAC corepressor complex to specific loci. The Sin3A–N-CoR–HDAC1/2 complex, for example, is recruited by unliganded nuclear receptors, the Mad family of basic helix–loop–helix zipper proteins, and p53 (64, 65, 126) (Fig. 5). SAP30, which binds to mSin3 and N-CoR, is required for N-CoR/mSin3-mediated repression of hydroxytamoxifen-bound estrogen receptor (ER) and homeodomain protein Rpx, but not unliganded retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (127, 128) (Fig. 4). Interestingly, microinjection of anti-N-CoR or anti-SMRT IgG into cells can convert hydroxytamoxifen-bound ER and RU486-bound progesterone receptor from repressors to activators (129). Further, there is intriguing evidence that decreasing the levels of N-CoR can lead to tamoxifen-resistance in breast cancer (129).

BRCA1 was shown to bind to RbAP46, RbAp48, HDAC1, and HDAC2, suggesting that BRCA1 may be a component of one or more of the HDAC1/2 multiprotein complexes (130). BRCA1 functions as a transcriptional coac-

long

tivator that associates with the RNA polymerase II holoenzyme, and is also involved in transcription-coupled DNA repair. Thus, BRCA1 may recruit HDAC complexes to sites of transcription and repair.

Transcription factors recruiting the class II HDACs are becoming known. Human myocyte enhancer factor 2 (MEF2) recruits HDAC4, resulting in the repression of MEF2 transcriptional activation. MEF2 also binds to the corepressor MEF-2 interacting transcription repressor (MITR), which shares sequence similarity with class II HDAC family members. MITR binds to HDAC1; thus, MEF2 is able to recruit both class I and class II HDACs (131). Similar to the situation with E2F1, MEF2 associates also with the coactivator/HAT CBP/p300. Thus, the association of MEF2 with corepressors or coactivators governs the transcriptional response of MEF2 (132).

4. ROLE OF HDACs IN TRANSCRIPTION

HDAC has a principal role in transcription repression (64). Once recruited to a specific promoter, HDAC deacetylates histones in nucleosomes, leading to the condensation of chromatin (65). However, acetylated HMG proteins and transcription factors may also be targets of the HDAC activity.

The HDAC corepressor complex can also repress transcription by mechanisms that do not require deacetylation. N-CoR and mSin3A of the HDAC complex interact with components of the preinitiation complex. Thus, the HDAC complex may interfere with the generation of a functional initiation complex (Fig. 4).

5. HATs, HDACs, AND CANCER

In humans loss of one allele of CBP is the underlying defect in Rubenstein-Taybi syndrome. Patients with this syndrome are more prone to cancer, consistent with the suggestion that CBP/p300 may function as a tumor suppressor. Further somatic translocations involving the *CBP* gene are found in various types of hematological malignancies (133, 134).

PML-RAR α , PLZF-RAR α , and AML-1-ETO, oncoproteins in acute promyelocytic leukemia (PML) generated by chromosomal translocations, recruit SMRT-mSin3A-HDAC1 and N-CoR-mSin3A-HDAC1/2 complexes (135-137). The SMRT-mSin3A-HDAC1 complex is recruited by the BTB/POZ domain found in the oncoprotein LAZ3/BCL6 (46). The recruitment of HDAC1 is crucial to the transforming potential of these oncoproteins. Inhibiting the HDAC activity with new-generation HDAC inhibitors appears to be a promising approach to the treatment of these cancers (135, 138).

6. LOCATION OF ACETYLATED HISTONES

The exact role of histone acetylation in transcription has been questioned for several decades. Researchers have accumulated substantial amounts of indirect evidence from chromatin fractionation and pulse chase labeling

long

antibodies experiments to show a relationship between this modification and transcription. Evidence elucidating the direct involvement of histone acetylation in transcription has also been obtained by the chromatin immunoprecipitation (CHIP) assay. In this assay, cells are fixed with formaldehyde, sonicated, and then regions of DNA associated with histones are isolated by immunoprecipitation with ~~antibodies~~ recognizing specific modified histone isoforms (139, 140). The use of this assay with an antibody that recognizes the ϵ -acetyllysine residues of all acetylated core histones showed that histone hyperacetylation corresponds to DNase I-sensitive, transcriptionally active and competent but not inactive regions of the chicken β -globin domain (52). Such a finding suggested that histone acetylation functions to maintain an open chromatin structure within transcriptionally active or competent genes, thereby increasing the access of transcription factors to these specific DNA target sequences. However, the antibody used in this study detected all acetylated histone isoforms. Therefore, the results of this study could not identify the role of specific acetylated histone isoforms in transcription. Elucidating the role of acetylated histone isoforms in transcription is important due to the recent discovery that several transcriptional cofactors containing intrinsic HAT or HDAC activity are targeted to specific histone substrates.

Realizing this, several investigators have produced antibodies specifically directed against different acetylated histone isoforms such as H4 acetylated at K5, K8, K12, and K16 (54) and H3 acetylated at K9 and K14 (141). Antibodies recognizing specific H4 acetylation sites have also been produced to further understanding of the role of site-specific acetylation in transcription (142). The use of these various antibodies in CHIP assays has led to several important observations. In one study, antiacetylated H3 and H4 antibodies were used in a CHIP assay to further define the role of insulator DNA sequences in transcription. The results of this study showed that insulator DNA sequences prevent histone deacetylation, and a loss of transcriptional activity in transgene expression (143). In addition, the CHIP assay has shown that transcriptional repression by proteins such as the Ikaros lineage-determining factor correlates with a reduction in H3 acetylation at the promoter of genes involved in lymphocyte development (144). Likewise, CHIP studies have shown that transcriptional silencing is correlated with a decrease in H3 and H4 hyperacetylation in yeast (54, 145). As well, the CHIP assay has shown that the nature of active and inactive gene structure in *X. laevis* is such that transcriptionally active, somatic genes are packaged with hyperacetylated H4, whereas transcriptionally inactive oocyte genes are packaged with hypoacetylated H4 (146). Similarly, a study on transgene expression in zebrafish demonstrated a correlation between H4 acetylation and transgene expression (147).

In another study, antiacetylated H3 and H4 antibodies were used to immunoprecipitate endogenous DNA sequences associated with the estrogen

receptor when bound to estrogen and antiestrogen ligand (90). This assay showed that the binding of estrogen to its receptor caused an induction of histone hyperacetylation at the promoter of several estrogen receptor target genes, ~~whereas the binding of the antiestrogen tamoxifen to the estrogen receptor target genes~~/whereas the binding of the antiestrogen tamoxifen to the estrogen receptor caused a dramatic reduction in histone acetylation at these same target genes (90) (Fig. 4). Contrary to the acetylation events caused by ligand-activated estrogen receptor, the CHIP assay with antibodies to anti-acetylated H4 and H3 has shown that genes (e.g., thyroid-stimulating hormone α) negatively regulated by the TR require the recruitment of an unliganded TR along with transcriptional corepressors for transcriptional stimulation and histone acetylation (148). As well, binding of ligand to the TR causes transcriptional repression along with a decrease in histone acetylation at the promoter of target genes (148). Thus, the CHIP assay has recently become an important tool for studying *in vivo* hormone-receptor action in transcription.

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The CHIP approach has also been used to determine the involvement of histone acetylation in events related to cell cycle and development. Immunoprecipitation of DNA sequences associated with antiacetylated H3 antibodies has shown that the retinoblastoma protein represses the transcription of cell cycle genes containing E2F sites by recruiting HDACs to the promoter, where they are able to deacetylate associated H3 (149). In addition, the FMR1 gene, a gene mutated in people with fragile X mental retardation, shows higher levels of H3 and H4 acetylation in cells from normal individuals when compared to cells from fragile X patients (150). The CHIP assay has also shown a link between signal-regulated H4 hyperacetylation events and transcription (151). More specifically, the hyperacetylation of H4 positioned along a reporter gene is induced by extracellular signals such as growth factor, stress, and cdc42 (151). The evidence accumulated from various CHIP studies combined with the identification that several transcription factors contain intrinsic HAT activity suggests that histone acetylation is directly and more actively involved in the transcriptional process than previously postulated.

The CHIP assay has also become a useful approach for fine-mapping the distribution of hyperacetylated histones along a gene. To fine-map histone isoforms to particular DNA sequences, nuclear lysates are extensively sonicated into fragments of approximately 350–500 bp in average size (141, 152). This allows the resolution of histone hyperacetylation over a range of 1 to 3 nucleosomes. Following this, acetylated histone isoforms along with their cross-linked DNA sequences are immunoprecipitated and the DNA is analyzed for particular sequences using quantitative polymerase chain reaction (PCR). To date, the results of several fine-mapping studies of histone hyper-

acetylation along transcriptionally active DNA regions show that the promoter region of a transcriptionally active gene is enriched in hyperacetylated histones H3 and H4, whereas the coding regions and regions upstream of the promoter are depleted in hyperacetylated histones (103, 141, 153). In yeast, the Sin3–Rpd3 HDAC complex causes histone deacetylation over a range of 1 to 2 nucleosomes within the promoter of a repressed gene (141). Similarly, transcriptional activation of the human interferon gene by virus infection induces histone hyperacetylation over 2 to 3 nucleosomes within the promoter region (103). Furthermore, the yeast Gcn5 HAT complex acetylates histones only in the HO gene promoter (153). Based on these studies, researchers postulate that HATs and HDACs undergo a targeted recruitment to the promoter. When at the promoter, these enzymes modify the acetylation state of the histones within a limited number of nucleosomes positioned on the promoter. This, in turn, causes localized perturbations in chromatin structure that enable transcription factors to gain access to their target DNA sequences.

Although the studies mentioned above provide a strong argument for promoter-targeted histone acetylation, conflicting data exist to suggest that HATs and HDACs are recruited to both the promoter and coding regions of transcriptionally active genes. First, a 60-kDa subunit of the elongator/RNA polymerase II holoenzyme referred to as Elp3 has been identified as a HAT and is able to acetylate all four core histones *in vitro* (63). Second, histone hyperacetylation is required to maintain the transcriptionally active nucleosome in an open conformation for transcriptional elongation (57). As well, when an antibody recognizing all acetylated histone isoforms is used in a CHIP assay, analysis of the DNA sequences associated with the immunoprecipitated histones shows a widespread distribution of histone hyperacetylation along the transcriptionally active *c-myc* and β -globin genes (52, 98). Thus, the distribution of acetylated histones or acetylated lysine residues within a gene may not be uniform (98). More specifically, the hyperacetylation observed throughout the *c-myc* and β -globin coding regions may represent hyperacetylated isoforms of H2B, as well as the hyperacetylated isoforms of H3 and H4 that are not recognized by the currently used antiacetylated H3 and H4 antibodies.

This contradiction in experimental evidence also suggests that a cell may contain two types of HATs with respect to the transcriptional process: those involved in transcriptional initiation, and those involved only in transcriptional elongation. HATs required for the initiation process would most likely function to alter the chromatin structure of the promoter, making the DNA more accessible to transcriptional initiation factors, whereas HATs required for elongation would increase the accessibility of elongation factors to the DNA within coding regions. In support of this, the p300 HAT interacts specif-

ically with the form of RNA polymerase II involved in transcriptional initiation, whereas the PCAF is associated only with the RNA polymerase II form involved in transcriptional elongation (154).

Cell type and the type of gene studied are also important factors that influence the distribution of acetylated histone isoforms within a cell's genome. The HDAC Rpd3 preferentially deacetylates H4 at K5 (155). This preference, however, is evident for only a select number of genes. In addition, the types of HATs and HDACs that target histones within the promoter of a gene can vary with cell type (156). Thus, the differences in cellular context between cell types most likely have a significant influence on the types of HATs and HDACs recruited to specific promoters.

Considering these studies, it is evident that the CHIP assay has made a significant contribution toward our understanding of the involvement of histone acetylation in various nuclear and cellular events.

F. Histone Methylation

The core histones H2B, H3, and H4 are modified by methylation. Heat shock of *Drosophila melanogaster* Kc cells induces methylation of H2B at an N-terminal proline residue (157). With the exception of plants, H4 is methylated at K20 (158) (Fig. 1). K20 of mammalian H4 is 70–100% methylated at this site. H3 may be methylated at K4, K9, K27, and K36, but the site utilization varies. Mammalian H3 is typically methylated at K9 and K27, being modified to 35 and 70–100%, respectively (159, 160). Cycad, *Chlamydomonas*, and *Tetrahymena* H3 are methylated at K4 to 20, 81, and 50%, respectively. Cycad and *Chlamydomonas* H3 are also methylated at K9, K27, and K36 but to varying extents (e.g., K9, 100 versus 16%; K27, 50 versus 25%). *Tetrahymena* H3 is methylated at K27 (40%) but not at K9 or 36. Chick H3 is methylated at K9, K27, and K36 to 20, 100, and 20%, respectively.

Acetylated isoforms of H3 and H4 are often the targets of ongoing methylation (161–164). In chicken immature erythrocytes, rapidly acetylated and deacetylated H3 and H4 are selectively methylated, whereas in HeLa cells dynamically acetylated H3, but not H4, is methylated (164, 165). H4 that is slowly acetylated and deacetylated is methylated in HeLa (164). The processes of histone methylation and dynamic acetylation are not directly coupled; neither modification predisposes H3 or H4 to the other (165). The association of dynamically acetylated histones with transcribed chromatin suggests that methylated H3 and (in some cases) methylated H4 are bound to transcriptionally active DNA (161, 164).

Histone methylation is a relatively stable modification with a slow turnover rate. However, there is evidence of methyl group turnover for HeLa H3 (164). It remains to be shown if this histone demethylase activity is present in transformed but not normal cells. Very little is known about the

histone methyltransferases. Histone-lysine methyltransferase is a chromatin-bound enzyme that catalyzes the addition of methyl groups onto the ϵ -amino groups of chromatin-bound H3 and H4 (161).

G. Histone Phosphorylation and Mitosis

The core histones and H1 undergo phosphorylation on specific serine and threonine residues. H1 can be phosphorylated on Ser/Thr residues on the N-terminal and C-terminal domains of the molecule, and H3 can be phosphorylated on Ser/Thr residues on its N-terminal domain. The phosphorylation of both H1 and H3 is cell cycle dependent, with the highest level of phosphorylation of both histones occurring in M phase. In G_1 phase of the cell cycle, the lowest number of H1 sites is phosphorylated, and there is a gradual increase in the number of sites phosphorylated throughout S and G_2 phases of the cell cycle. In M phase, when chromatin is highly condensed, the maximum number of sites is phosphorylated. The strong correlation between highly phosphorylated H1 and chromatin condensation at mitosis leads to the assumption that H1 phosphorylation drives mitotic chromatin condensation; however, chromatin condensation can occur in the absence of H1 phosphorylation (166). H1 phosphorylation destabilizes chromatin structure and weakens its binding to DNA. Therefore, H1 phosphorylation may lead to decondensation of chromatin and access of the DNA to factors involved in transcription and replication in G_1 and S and to condensing factors present in mitosis (167 and references therein).

Studies on H3 phosphorylation during mitosis have revealed that Ser-10 phosphorylation of H3 is correlated with both mitotic and meiotic divisions in *Tetrahymena* micronuclei (168), and that phosphorylation at this site is required for proper chromosome condensation and segregation (169). In mammalian cells, mitosis-specific phosphorylation of H3 on Ser-10 initiates primarily within pericentromeric heterochromatin during late G_2 and spreads in an ordered fashion throughout the condensing chromatin and is complete just prior to the formation of the prophase chromosomes (170). Phosphorylation of H3 at Ser-10 weakens the association of the H3 tail to DNA, which may promote the binding of factors that drive chromatin condensation as cells enter mitosis (171). Phosphorylation of H3 at Ser-28 was shown to occur in mammalian cells during early mitosis, suggesting that H3 phosphorylation at sites Ser-10 and Ser-28 are involved in mitotic chromosome condensation (172).

H. Histone Phosphorylation, Transcription, and Signal Transduction

Studies by Lee and Archer show an involvement of H1 phosphorylation in gene transcription. Inactivation of the mouse mammary tumor virus (MMTV) promoter is associated with dephosphorylation of H1, and reacti-

vation of the promoter is associated with rephosphorylation of H1 (173). Further, mouse H1b phosphorylation is dependent on ongoing transcription and replication processes; the inhibition of these processes may alter accessibility of H1b to the H1b kinase, which would result in decreased levels of phosphorylated H1b (174). The modification of this mouse histone is unique in this regard. No other histone modification has been shown to be dependent on these processes.

Oncogene-transformed mouse fibroblasts have a more decondensed chromatin structure than do parental cell lines (167). We found that levels of phosphorylated H1 were elevated in oncogene (*ras*, *raf*, *fes*, *mos*, *myc*) and aberrantly expressed MAPKK (MEK) transformed mouse fibroblasts, which have elevated activities of MAPK (ERK1 and ERK2) (167) (Fig. 6). Subsequently,

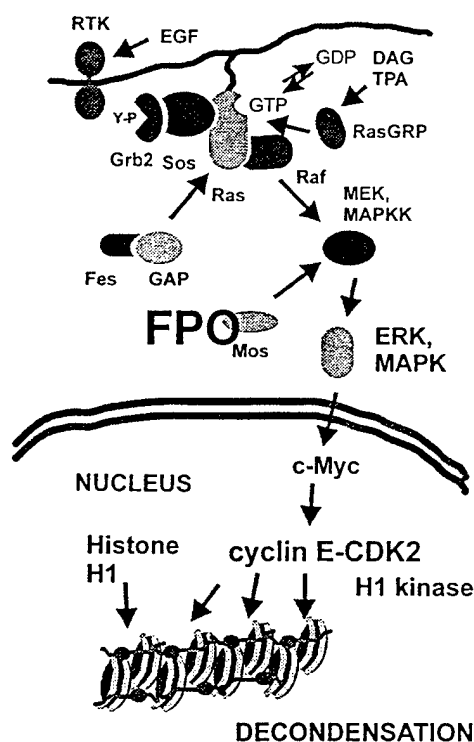


FIG. 6. Activation of the Ras-MAPK signaling pathway increases the level of phosphorylated H1. Mitogen-activated protein kinase kinase (MAPKK) and mitogen-activated protein kinase (MAPK) are also called MEK and ERK, respectively. Activation of ERK leads to the increased activity of cyclin E-cdk2, an H1 kinase. H1 phosphorylation results in decondensation of chromatin.

Weinberg's group found that *Rb*-deficient human fibroblasts have increased levels of phosphorylated H1 and a relaxed chromatin structure (175). These authors had evidence that cyclin E/cyclin-dependent kinase 2 (cdk2) was directly involved in increasing the levels of phosphorylated H1. Our unpublished results also suggest that elevated cyclin E-associated H1 kinase (cdk2) activity resulting from persistent activation of the Ras-MAPK pathway is responsible for the increased level of phosphorylated H1 in oncogene-transformed mouse fibroblasts (D. N. Chadee, C. P. Peltier, and J. R. Davie, unpublished observations). Persistent activation of the Ras-MAPK signaling pathway also results in elevated levels of phosphorylated (Ser-10) H3 in oncogene-transformed mouse fibroblasts (Fig. 7). The remodeling of chromatin structures resulting from increased H3 phosphorylation may contribute to aberrant gene expression (176).

Though most studies on H3 phosphorylation have focused on the phosphorylation that occurs in mitosis, H3 phosphorylation also occurs in G_1 .

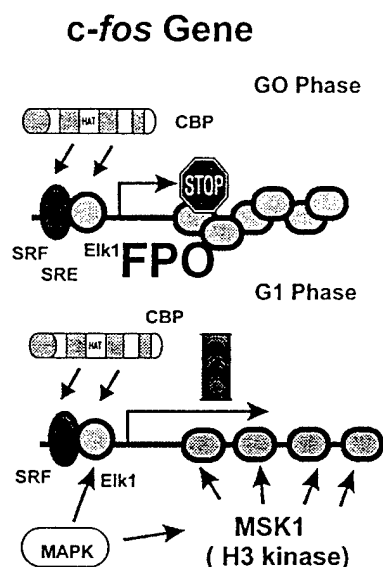


FIG. 7. Regulation of the expression of the immediate-early *c-fos* gene. In G_0 phase cells, the serum response element (SRE) of the *c-fos* promoter is loaded with transcription factors SRF and Elk1, which recruit the coactivator/HAT CBP. Stimulation of the cells with growth factors or phorbol esters results in the activation of the Ras-MAPK pathway and activation of MAPK/ERK. MAPK phosphorylates the SRE bound transcription factors, CBP and MSK1, an H3 kinase. CBP would acetylate the nucleosomal histones and MSK1 would phosphorylate H3. The net result of these modifications would be the decondensation of chromatin and the release of the elongation block.

Phosphorylation of H3 has been implicated in the establishment of transcriptional competence of immediate-early response genes. H3 is rapidly phosphorylated when the Ras-MAPK pathway of serum-starved cells is stimulated with growth factors and phorbol esters. H3 phosphorylation is concurrent with the transcriptional activation of the early response genes *c-fos* and *c-jun* (176). We demonstrated that the newly phosphorylated H3 is located in numerous small foci scattered throughout the interphase nuclei of 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated cells; the foci were found outside condensed chromatin regions (176). Highly acetylated H3 is also observed in similarly positioned numerous small foci, which agrees with the observation that H3 phosphorylation is restricted to a small fraction of H3 histones that are dynamically highly acetylated (6). Using the CHIP assay, we provided direct evidence that the newly phosphorylated H3 is associated with induced *c-fos* and *c-myc* genes (176). The observation of numerous foci of newly phosphorylated H3 in TPA-treated cells suggests that many other induced genes, such as those described by Brown and colleagues, are associated with phosphorylated H3 (177).

The *c-fos* gene is transcribed in quiescent cells; however, elongation of the gene is blocked approximately 100 nucleotides from the site of initiation. Stimulation of the Ras-MAPK pathway results in the release of this block in elongation (Fig. 7). Activation of the MAPK signaling pathway results in the phosphorylation and activation of transcription factors, such as the Ets transcription factor family (178). The *c-fos* promoter serum response element (SRE) is continuously occupied by SRF and Ets proteins of the TCF family; both of these factors are targets of signaling pathways (178). p62 TCF and Elk-1 (members of the Ets family) are direct targets of the Ras-MAPK signaling pathway and are phosphorylated by MAPK (p42/p44; also named ERK1/2) (179, 180). Phosphorylation of the Ets proteins is thought to contribute to the induced expression of the *c-fos* gene. CBP binds to both Elk-1 and SRF (179). Elk-1 phosphorylation results in a functional interaction between Elk-1 and CBP (181). Thus, these transcription factors recruit a coactivator with HAT activity. It is possible that phosphorylation and, likely, acetylation of H3 associated with the *c-fos* gene are also involved in release of the elongation block, by allowing the chromatin fiber to be less compact. Consistent with this hypothesis, the *c-fos* chromatin becomes more DNase I sensitive following activation of the Ras-MAPK pathway (182). As the H3 tail contributes to the folding and interassociation of chromatin fibers, modification of the H3 tail by acetylation and phosphorylation may destabilize higher order compaction of the chromatin fiber and contribute to maintaining the unfolded structure of the transcribing nucleosome.

The steady-state level of H3 phosphorylation is dependent on a balance of phosphatase and kinase activities in the cell. Protein phosphatase 1 appears

to be the H3 phosphatase (176). Allis and colleagues have presented evidence that the activity of Rsk2, a member of the pp90^{msk} kinases, is required for the mitogen-stimulated phosphorylation of H3 (183). Coffin-Lowry patients have a mutation in the Rsk2 gene. Fibroblasts from these patients do not exhibit epidermal growth factor (EGF)- or TPA-stimulated phosphorylation of H3 and, interestingly, growth factor-induced expression of the *c-fos* gene is severely impaired. However, Mahadevan and colleagues presented evidence that MSK1 is the H3 kinase (184). Both Rsk2 (MAPKAP kinase-1 β) and MSK1 are members of a subfamily of MAPK-activated protein kinases with two distinct protein kinase domains. MSK1, but neither ERKs nor Rsk2, is inhibited by H89, a protein kinase inhibitor. H89 inhibits TPA- and EGF-stimulated H3 phosphorylation and expression of *c-fos* and *c-jun* (184). In our *in vitro* studies, we found that Rsk1 and Rsk2 efficiently phosphorylated H2B, which has two Rsk consensus sequences (RXXS), but failed to phosphorylate H3, which has an RXS sequence at Ser-10 and Ser-28 (D. N. Chadee, C. P. Peltier, I. S. Sterlkov, J. R. Davie, unpublished observations). Our data concur with those of Mahadevan and colleagues that Rsk2 is not the physiological relevant TPA- or EGF-stimulated H3 kinase.

V. Karyoskeleton and Organization of Chromatin

The chromatin fiber is organized into loops such that the base of the loop is attached to proteins of the karyoskeleton (also called nuclear matrix and nucleoskeleton) (28, 185, 186). The DNA sequence binding to the karyoskeleton is called the matrix attachment region (MAR) or scaffold attachment region (SAR). The S/MARs delineate the loop domain in different cell types regardless of the transcriptional activity of the gene(s) within the domain. The loop domain containing transcribed genes has a less condensed, more DNase I-sensitive structure than that of loops with repressed genes. For transcriptionally active chromatin loops, the boundaries of the several DNase I-sensitive gene domains comapped with S/MARs (28). A comparison of the DNA sequences of S/MARs shows that they do not share extensive sequence homology; however, S/MAR DNA sequences have high bending potential and may act as topological sinks (187).

The karyoskeleton is the nuclear structure that is present following the salt extraction of nuclease-digested nuclei (188). It is the dynamic structural framework of the nucleus composed of a meshwork of core filaments linked to the nuclear lamina proteins. The diameters of core filaments of the internal matrix are similar to diameters of cytoskeletal intermediate filaments (188, 189). The composition of the core filaments is not yet known (190). However, there is an interesting observation that ~~the nuclear matrix~~ NuMA is capable of forming a scaffold (191).

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There has been much controversy over the existence of the karyoskeleton. The rather harsh methods (high salt extraction of nuclease-digested nuclei) originally used to isolate karyoskeletons were criticized as generating artifacts. However, additional studies have defused these concerns. Karyoskeletons isolated by 0.2 M ammonium sulfate extraction of DNase I-digested nuclei maintained the organization of nuclear components such as transcription sites and nuclear speckles, which are involved in storage of RNA splicing factors (27). Further formaldehyde fixation of cells or nuclei followed by isolation of karyoskeletons yielded structures similar to unfixed karyoskeletons (192). More gentle methods in which fragmented chromatin is removed from nuclei at physiological ionic strength yielded karyoskeleton structures essentially identical to those prepared by more harsh methods (26). A study of major importance was published by Hendzel and colleagues, in which they visualized the karyoskeleton fiber network in cells (193).

VI. Karyoskeleton and Transcription Factories

The karyoskeleton has a central role in transcription (26, 27). There are about 2000 transcription sites in a HeLa nucleus, with each of these sites containing about 25 active polymerases and greater than 10 active genes (194). These sites are referred to as transcription factories. The transcription machinery is associated with the karyoskeleton. Using a gentle extraction method, Roeder, Cook and colleagues presented evidence that active RNA polymerases are attached to the karyoskeleton (26). Cells were lysed with saponin in a physiological buffer, and the release of transcription factors and RNA polymerase II into soluble and insoluble cellular fractions were analyzed. RNA polymerase II was observed in both fractions, with unengaged RNA polymerase II (predominantly form IIA) being found in the soluble fraction and engaged RNA polymerase II (predominantly form IIO) being present in the insoluble (karyoskeleton) fraction.

VII. Transcriptionally Active Chromatin and the Karyoskeleton

Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus (28, 195). Actively transcribed, but not inactive, chromatin regions are immobilized on the nuclear matrix by multiple dynamic attachment sites. When histones are removed by high salt, loops of DNA are seen emanating from a central nuclear skeleton, forming a halo around this nuclear structure. Transcriptionally inactive genes are found in the halo, whereas DNA loops with transcriptionally active genes

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remain associated with the residual nucleus (186, 195). The transcription machinery, transcription factors, and nuclear enzymes are thought to mediate the dynamic attachments between transcribing chromatin and nuclear matrix.

VIII. Transcription Factors and the Karyoskeleton

The karyoskeleton has a role in the expression of genes by concentrating a subset of transcription factors at specific nuclear sites (196, 197). Transcription factors associated with the karyoskeleton include estrogen receptor, mutant p53, YY1, AML-1, Sp1, and Rb (198–201). The karyoskeleton targeting sequence (nuclear matrix targeting sequence, or NMTS) has been identified for several factors, including AML-1, YY1, glucocorticoid receptor, and Pit-1 (196, 201–204). At present there is no consensus sequence or structure for the NMTS of these factors. For AML-1, the factor's NMTS functions as a transactivation domain and targets AML-1 to karyoskeleton sites containing a hyperphosphorylated active form of RNA polymerase II (196).

The association of a transcription factor with the karyoskeleton is dynamic (201, 204), and the equilibrium between karyoskeleton bound and unbound states may be influenced by protein modification. For example, hypophosphorylated Rb associates with the karyoskeleton only during early G₁ phase of the cell cycle. Throughout the remainder of the cell cycle Rb becomes phosphorylated and is dephosphorylated in late mitosis. Thus, the association of Rb with the karyoskeleton may be determined by phosphorylation.

Identification of the karyoskeleton acceptor for the various transcription factors is currently under investigation. Several karyoskeleton acceptors for hypophosphorylated Rb have been reported. Durfee *et al.* identified an 84-kDa karyoskeleton Rb acceptor that binds to the N-terminal region of Rb, in monkey kidney CV-1 cells (205). The 84-kDa protein colocalized with B1C8 to speckles or interchromatin granule clusters. NRP/B (nuclear restricted protein/brain), which is expressed in primary neurons, is also a karyoskeleton acceptor for hypophosphorylated Rb (206).

The karyoskeleton has a central role in steroid hormone action (207, 208). The estrogen receptor (ER) is associated with the karyoskeleton of estrogen-responsive tissues (209–212). In *in vitro* reconstitution studies with karyoskeletons and hormone receptors (e.g., ER and androgen receptor), it has been shown that nuclear acceptor sites for the hormone receptors are associated with the karyoskeleton (207, 209, 213) (Fig. 4). The binding of ER to the karyoskeleton was saturable, of high affinity, target tissue specific, and receptor specific (209). In studies with estrogen-responsive tissues, including human breast cancer cell line MCF-7, evidence for the presence of ER acceptor proteins has been reported (207, 214). However, we do not know the identity of the karyoskeleton acceptor proteins for ER in human cells. Bind-

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ing studies with ER provide evidence that some karyoskeleton acceptors for ER are ligand dependent (214).

To observe ER subcellular trafficking and localization in living cells, we tagged the N terminus of ER with the S65T variant of the green fluorescent protein (GFP) (212). The GFP-ER fusion protein was a functional protein, as determined by transient transfection assays. Independent of ligand, GFP-ER protein was found in the nucleus of human breast cancer cells, with the exception of ICI 182780, with which some cytoplasmic GFP-ER was observed. In ligand-free conditions, a diffuse pattern of nuclear localization was seen for GFP-ER in MCF-7 cells. When estradiol was added to the media, the GFP-ER pattern became more punctate or speckled. The same effects were observed when 4-hydroxytamoxifen and ICI 182780 were added. Transiently expressed GFP-ER was associated with the nuclear matrix. We are currently testing the idea that the GFP-ER nuclear patterns reflect the location of nuclear matrix acceptors for ER.

IX. HATs, HDACs, and the Karyoskeleton

Both HATs and HDACs are associated with the karyoskeleton (64). We proposed that karyoskeleton-associated HAT and HDACs mediate a transient attachment of transcriptionally active chromatin to the karyoskeleton. Many of the transcription factors that associate with the HATs or HDACs are karyoskeleton associated (e.g., YY1, hypophosphorylated Rb, Sp1, GATA-1, ER) (215) (Figs. 4 and 5). HATs (e.g., TAF_{II}250, CBP), HDACs, and dynamically acetylated histones are found gathered around interchromatin granule clusters (6). The karyoskeleton may have a role in establishing this organization. Importantly, these interactions between HATs, HDACs, transcription factors, and active chromatin should be viewed as dynamic.

We reported that HDAC1 is associated with matrix-associated region DNA in human breast cancer cells (185). These results suggest that HDAC1 may have a role in the organization of nuclear DNA. It is interesting to note that the attachment-region binding protein (ARBP), a karyoskeleton protein that binds to S/MARs, is homologous to MeCP2 (216). Thus, the N-CoR-Sin3A-HDAC1 complex could be recruited to the karyoskeleton and to S/MAR DNA by MeCP2/ARBP (Fig. 5).

X. Mechanical Signaling Pathways and Organization of Nuclear DNA

There is ample evidence that the tissue matrix system, which consists of the extracellular matrix, cytoskeleton, and karyoskeleton, sends signals from the cell exterior to the nuclear interior (217-219). Changes in the shape of the nucleus and the cytoskeleton most likely alter chromatin structure and

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perturb the karyoskeleton. Changes in cell and nuclear shape regulate cell proliferation and gene expression (220, 221).

A series of molecular events are put into play when a cell receives suitable extracellular cues to go from quiescence to a proliferative state. Activation of the Ras-MAPK pathway is necessary, but not sufficient, for cells to proliferate. Alterations in cell shape as defined as degree of cell extension or spreading are also required for cells to enter the cell cycle (220, 222, 223). The initial transient activation of the Ras-MAPK pathway results in the expression of p21^{cip1}, an inhibitor of cyclin E-cdk2 (224). Cell adhesion to the ECM is required for the sustained activation of the Ras-MAPK pathway and expression of cyclin D1 (220, 222, 225). The cytoskeleton, particularly the actin network, plays a pivotal role in these events (220, 223). The active cyclin D1-cdk4/6 complex then phosphorylates Rb, resulting in the dissociation of HDAC (122). As cells reach middle to late G₁ phase of the cell cycle, the expression of p21^{cip1} declines. Cell adhesion to the ECM is required for down-regulation of p21^{cip1} and p27^{Kip1}, which are inhibitors of cyclin E-cdk2 (224). Following the reduction of p21^{cip1} and p27^{Kip1}, active cyclin E-cdk2 further phosphorylates Rb, preventing Rb from binding to E2F1. Phosphorylation of Rb also results in its liberation from the karyoskeleton. E2F-regulated genes, such as cyclin A, are then expressed. Cells have entered into S phase with activation of cyclin A-cdk2. Thus, chemical and mechanical signaling pathways are required for quiescent G₀ phase cells to move through G₁ phase of the cell cycle (220).

The β -casein gene is an excellent example of the importance of cell shape and structure in gene regulation. The extracellular matrix and prolactin activate the BCE-1 enhancer of this gene through at least three transcription factors binding to the enhancer and, perhaps, by altering the acetylation state of the histones. The chromatin context of the enhancer is critical because BCE-1 on a nonintegrated template will not respond to extracellular and prolactin signals (226). The authors proposed two mechanisms by which the extracellular matrix induces the expression of the β -casein gene. Changes in the three-dimensional architecture of the cell by the extracellular matrix could alter the three-dimensional structure of the nucleus and the structure and/or composition of the karyoskeleton. Perturbation in the karyoskeleton could reposition karyoskeleton-associated HATs and/or HDACs (227), resulting in the remodeling by histone acetylation and transcriptional activation of the β -casein chromatin template. Alternatively, the extracellular matrix could induce or modify cofactors that have HDAC or HAT activity (64). Thus this study shows how the structure of a cell can play a role in the regulation of gene expression.

Current evidence suggests that intermediate filaments relay signals from the plasma membrane to nuclear DNA, resulting in changes in chromatin organization and perhaps function. Intermediate filaments, a component of the

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cytoskeletal network, extend from the plasma membrane and penetrate the nuclear lamina (228–231). Further, intermediate filament proteins (cytokeratins and vimentin) are positioned to make contact with nuclear DNA *in vivo* (228, 232, 233).

Using the cross-linking agent *cis*-diamminedichloroplatinum (cisplatin), it has been shown that cisplatin preferentially cross-links karyoskeleton proteins to S/MAR DNA *in situ* (234). The interactions between intermediate filament proteins and nuclear DNA are dynamic. For example, the interaction between cytokeratin intermediate filaments and nuclear DNA is regulated by estrogens in ER-positive, hormone-dependent breast cancer cells (232, 235). Our studies suggested that estrogen regulated the levels of cytokeratins in the cells (232). In hormone-dependent cells estrogen-regulated interactions between intermediate filaments and nuclear DNA could manipulate the organization of chromatin. However, in ER-positive, hormone-nonresponsive breast cancer cell line T5-PRF, the estrogen regulation of cytokeratins with nuclear DNA was not observed (232, 235).

It was reported that activity of ERK1 and ERK2 was elevated in the T5-PRF cell line (236). ER is a phosphoprotein that may be phosphorylated at multiple sites by a variety of kinases. Stimulation of the RAS–MAPK pathway results in the phosphorylation of ER at Ser-167 and Ser-118 (237, 238). This phosphorylation event enhances DNA binding and transcription of ER (237, 238). Thus, enhanced activity of the Ras–MAPK pathway in the T5-PRF cell line may negate the requirement for estrogen. There is evidence that the cytokeratin genes may be estrogen responsive (239). The constitutive activity of ER in the T5-PRF cell line may therefore result in deregulated expression of the cytokeratin genes.

It is not known which DNA sequences are associated with the intermediate filaments and what are the consequences of the interaction between the cytoskeleton and chromatin. Because intermediate filaments are located at the periphery of the nucleus, they likely interact with heterochromatin. However, because transcribed genes are associated with the nuclear periphery, it is also possible that the intermediate filaments are in contact with transcribed regions of chromatin (240, 241). It was shown that a subset of karyoskeleton proteins may be involved in chromosome territory organization (242). Thus, the interactions between intermediate filaments, the karyoskeleton, and chromatin may have profound effects on the organization of chromosome territories and surrounding nuclear components.

XI. Future Directions

In the past 4 years, we have seen major breakthroughs in our understanding of the relationships between histone-modifying enzymes, transcription,

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and signal transduction pathways. Future studies will further illuminate the role of the chemical and mechanical (tissue matrix system) signaling pathways in altering chromatin structure and gene expression. How HATs and kinases activated by various signal transduction pathways are recruited and modify specific regions of chromatin will be realized. Many histone modifications occur in concert, e.g., mitogen-stimulated histone H3 phosphorylation and dynamic acetylation. Future studies will reveal the mechanisms by which HATs, HDACs, histone kinases, and other histone-modifying enzymes are recruited to specific sites in nuclear space. We believe that the karyoskeleton has an important role in this process. Transiently assembled huge multiprotein complexes assembled on the karyoskeleton could coordinately catalyze chromatin remodeling and transcription. There is still much to be learned about the function of the histone tails and the consequences of modifications on tail function. Further, there are several histone-modifying enzymes that remain to be purified and characterized, e.g., enzymes catalyzing histone methylation and demethylation. In understanding the function of the activated histone-modifying enzymes, we need to know the precise gene location of the modified histone isoforms. The CHIP technology with antibodies recognizing specific modified histone isoforms will be pivotal to learning which segments of a gene are targets of histone-modifying enzymes. Increasingly there are examples in the literature of how misdirecting and/or deregulating the activity of a histone-modifying enzyme leads to abnormal gene expression and cancer. In future studies, we will see the development of exciting new agents to inhibit the activity of these rogue enzymes, leading to novel approaches to treat cancer.

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